This number completes the Volume 43

Vol. 43, No. 6 **November, 1956**

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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PUBLISHED BIMONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo,
Japan

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PRINTED IN JAPAN

The Journal of Biochemistry was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this Journal has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This Journal is collecting the original investigations on the subjec in this country. It is published bimonthly, one volume per year

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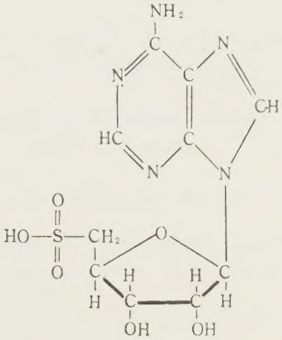
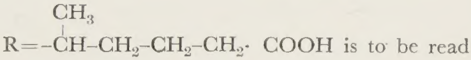
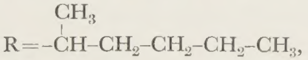
ERRATA

p. 32,	line 4	June is to be read July.
p. 93,	line 7	1956 is to be read 1955.
p. 95,	TABLE II	ER is to be read FR.
p. 96,	line 5	Bucher is to be read Buchner.
p. 156,	CHART	The structure of adenosine-5′-monosulfate is to be corrected as follows: 
p. 156,	line 8	FMN is to be read FMS
p. 311,	CHART	On the arrow from Dihydro derivative to 3,12-Dihydroxy-7-keto derivative, add as follows: $\xrightarrow[\text{alkaline}]{\text{Br}}$
		 $R=-\overset{CH_3}{\overset{\vert}{C}}H-CH_2-CH_2-CH_2\cdot\ COOH$ is to be read  $R=-\overset{CH_3}{\overset{\vert}{C}}H-CH_2-CH_2-CH_2-CH_3,$
		$A=CH_3O-$ is to be read $Ac=CH_3CO-$
p. 382,	line 8	$RnH_2+O_2\rightarrow Rn^*+H_2O$ is to be read $RnH_2+\frac{1}{2}O_2\rightarrow Rn^*H_2O$.
p. 382,	line 16	$RnH_2+H_2O\xrightarrow{\text{peroxidase}}Rn+2H_2O$ is to be read $RnH_2O_2\xrightarrow{\text{peroxidase}}Rn+2H_2O$.
p. 399,	line 5	1*st Surgical Clinic*, is to be added
p. 407,	line 8	Acknowledgement. Prof. Dr. H. Miyake is to be added.
p. 461,	line 15	$AgNO_2$ is to be read KNO_3
p. 476,	line 8	adjust is to be read adjusted.
p. 497,	line 9	rage is to be read range.
p. 623,	line 6	most is to be read mere.
p. 654,	line 7	afte is to be read after.
p. 663,	line 10	TABLE VI (2) is to be read TABLE IV (2).
p. 665,	line 18	C^{14}-glycine is to be read glycine.
p. 665,	line 20	1.27 μM is to be read 1.27 mM.
p. 666,	line 38	than the proteins is to be read than those into the proteins.
p. 667,	line 11	than that into other protein is to be read than those into those into other protein.

Notice to Subscribers

Beginning in January, 1957, *The Journal of Biochemistry* will be published **monthly**, starting from Volume 44, No. 1. (January) to No. 12 (December). It will cover about 900 pages of the papers of original investigations in this country. The *Subscription Price* is not changed ; ¥3,600 (U.S. $10.00) per volume, postage included.

The Journal of Biochemistry, Vol. 43, No. 6, 1956

STUDIES ON BACTERIAL AMYLASE

I. AMINO ACID COMPOSITIONS OF CRYSTALLINE BACTERIAL AMYLASE FROM B. SUBTILIS N

BY SHIRO AKABORI, YOSHIMI OKADA, SADAO FUJIWARA AND KIN-ICHI SUGAE

(From the Laboratory of Biochemistry, Faculty of Science, Osaka University)

(Received for publication, July 20, 1956)

Amino acid compositions of pancreatic amylase from swine (*1*), recently human salivary amylase (*2*) and Taka-amylase-A (*3*) have been reported. Bacterial amylase (α-type) has been first isolated in crystalline state by K. H. Meyer *et al.* (*4*). B. Hagihara (*5*) described an excellent method for the preparation of crystalline bacterial amylase from culture media of amylase producing bacteria belonging to *B. subtilis*. In this report, the results of the quantitative determination of all amino acids of crystalline bacterial amylase from *B. subtilis* N are described, and it is discussed whether or not there are any similarity in the amino acid compositions of various amylases.

EXPERIMENTS AND RESULTS

Materials—A sufficient quantity of crystalline bacterial amylase was generously supplied by Dr. B. Hagihara. The dry weight was determined on an aliquot by drying at 56° over phosphorous pentaoxide in vacuo. The dry protein contained 0.59 per cent ash, 16.23 per cent nitrogen and 0.23 per cent sulfur. No sugar was detected in the protein*.

Figures presented in this paper are all based on dry weight of protein.

Hydrolysis—The crystalline bacterial amylase was hydrolyzed with 20 times the weight of constant boiling hydrochloric acid at 100° for 24 hours. After the removal of the excess of hydrochloric acid in vacuo, the residual hydrolysate was taken up in a definite volume of proper solvent and used for analysis.

The determination of the cystine content of the protein is complicated by the instability of cystine and cysteine during hydrolysis.

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* The sugar determination was done by Mr. H. Hanafusa.

The authors employed the method of Schram *et al.* (*6*) which based upon the oxidation of the protein by performic acid, followed by hydrolysis with hydrochloric acid and determination of the resulting cysteic acid.

Analytical Method—Amino acid contents were determined by chromatography on the ion-exchange resin Dowex-50 columns according to the method of Moore and Stein (*7*). The buffer solution was prepared according to the direction given by the same authors (*7*) except that no benzylalcohol and no detergent were added. The column of 100 cm. in height was operated with control of constant temperature, which was at first at 37.5° and changed to 50° at the beginning of the valine peak, but not increased to 75°, since tyrosine and phenylalanine was separated satisfactorily at 50°. The shorter column was operated at room temperature.

No separation of glutamic acid and proline on the column of 100 cm. in height was achieved in our experiments. Proline content was determined according to the method of Moore and Stein using starch column chromatography (*8*). The solvent used was *n*-butanol-*n*-propanol-0.1 *N* hydrochloric acid (1 :2 :1).

Cystine plus cysteine was determined as cysteic acid on column of ion-exchange resin Dowex-2 according to the method of Schram *et al.* (*6*).

Tryptophan content was determined by ultra-violet absorption spectra of intact protein (*9*).

Analytical Results—Effluent curves obtained on 100 cm column are represented in Fig. 1 and the numerical values are shown in Table I.

The results of the analysis of basic amino acids are given in Fig. 2 and Table II.

This protein contained no cystine and cysteine. Effluent curve obtained on Dowex-2 column are shown in Fig. 3. The broken line represents the position correspond to cysteic acid.

The distribution of amino acids in effluent on starch column was similar to that reported by Stein and Moore (*8*). Proline content was 3.49 as residual per cent.

The ultra-violet absorption spectra of intact protein in 1/10 *N* sodium hydroxide solution are shown in Fig. 4. Tryptophan content calculated from absorption spectra was 5.65 as residual per cent.

All the results of the analysis described above are summerized in Table III.

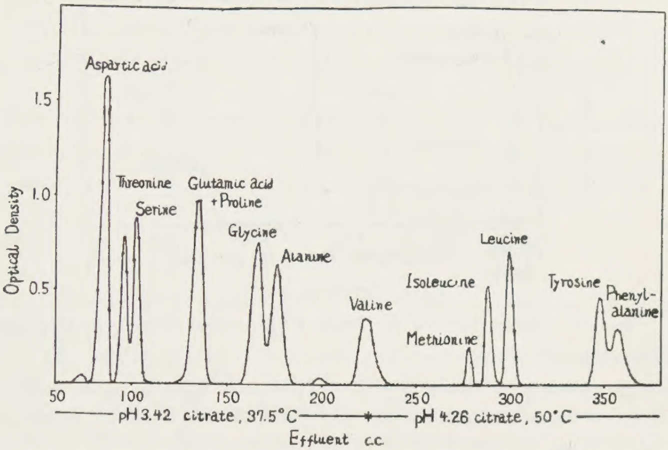

FIG. 1. Separation of amino acids from a hydrolysate of bacterial amylase.

The column of Dowex 50, 0.9 × 100 cm., was operated in the sodium form, with buffers of the pH and temperature indicated as eluants. A sample of 2.79 mg. of hydrolysate was chromatographed.

TABLE I

Chromatographic Analysis of Hydrolysates of Bacterial Amylase

The hydrolysate was chromatographed on Dowex 50 in the manner indicated in Fig. 1. 2.79 mg. of hydrolysates were employed for each chromatogram.

Chromatogram No.	3		4	
Amino acids	g. of amino acid per 100 g. of protein	N as per cent of protein N	g. of amino acid per 100 g. of protein	N as per cent of protein N
Aspartic acid	15.62	9.91	14.56	9.24
Threonine	6.31	4.48	6.09	4.32
Serine	6.11	4.98	5.74	4.61
Glutamic acid	13.46	7.72	12.12	6.95
Glycine	5.62	6.32	5.69	6.40
Alanine	6.02	5.71	5.41	5.09
Valine	5.78	4.17	5.38	3.88
Methionine	1.50	0.85	1.01	0.63
Isoleucine	4.12	2.65	3.83	2.46
Leucine	6.28	4.04	6.57	4.23
Tyrosine	8.33	3.88	8.29	3.87
Phenylalanine	7.05	3.60	5.85	2.99

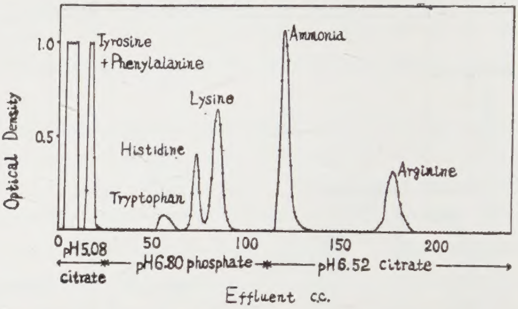

FIG. 2. Separation of the basic amino acids from a hydrolysate of bacterial amylase.

The column of Dowex 50, 0.9 × 15 cm., was operated in the sodium form at the room temperature, with the buffers indicated. A sample of 2.79 mg. of hydrolysate was chromatographed.

TABLE II

Chromatographic Analysis of Basic Amino Acids of Bacterial Amylase

The hydrolysate was chromatographed on Dowex 50 in the manner indicated in Fig. 2. 2.79 mg. of hydrolysates were employed for each chromatogram.

Amino acid	g. of amino acid per 100 g. of protein	N as per cent of protein N
Histidine	3.80	6.20
Lysine	7.30	8.41
Ammonia	1.45	7.20
Agrinine	6.78	13.17

DISCUSSION

Bacterial amylase of *B. subtilis N* distinguishes itself from other proteins, except gelatin (*10*) and myoglobin (*10*, *11*), by the absence of cystine and cysteine. The sulfur content of 0.23 per cent found by elemental analysis is in agreement with the value of 0.27 per cent obtained from methionine content. Another salient feature of the amino acid composition is relatively high contents of aspartic and glutamic acids, tryptophan and tyrosine, and low contents of methionine.

Amino acid compositions of four amylases analysed heretofore i.e., pancreatic amylase from swine (*1*), human salivary amylase (*2*) Taka-

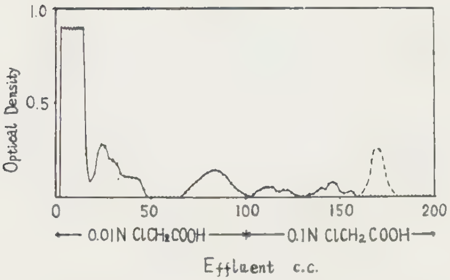

Fig. 3. Chromatogram of a hydrolysate of oxidized bacterial amylase.

The column of Dowex 2 (0.9 × 15 cm.) was operated using aqueous chloroacetic acid. The sample corresponded to 15.1 mg. of bacterial amylase. The broken line represents the position corresponding to cysteic acid.

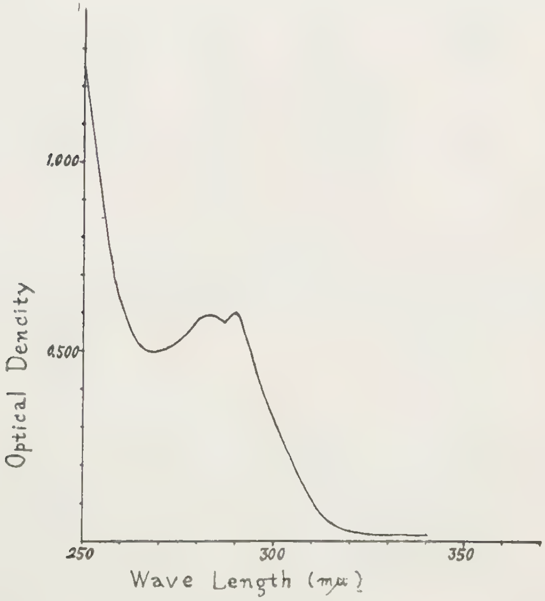

Fig. 4. The ultra-violet absorption spectrum of bacterial amylase. The concentration is 2.307 mg. per 10 ml. and the determination was done in 0.1 N sodium hydroxide solution.

TABLE III

Amino Acid Composition of Bacterial Amylase

Amino acids	g. of amino acid per protein	g. of amino acid residue per 100 g. of protein	N as per cent of protein N	Moles of amino acid per 10^5 g. of protein
Glycine	5.64	4.30	6.36	75.2
Alanine	6.02	4.80	5.71	67.6
Valine	5.55	4.66	4.00	47.4
Leucine	6.42	5.54	4.13	49.0
Isoleucine	3.97	3.42	2.55	30.3
Proline	4.14	3.49	3.04	36.0
Phenylalanine	5.85	5.22	2.99	35.4
Tyrosine	8.31	7.48	3.87	45.8
Tryptophan	6.19	5.65	5.24	30.4
Serine*	6.24	5.17	5.05	59.5
Threonine*	6.36	5.40	4.51	53.4
Cystine+Cysteine	0	0	0	0
Methionine	1.26	1.11	0.73	8.5
Arginine	6.78	6.08	13.17	38.9
Histidine	3.80	3.38	6.20	24.5
Lysine	7.30	6.40	8.41	49.9
Aspartic acid	15.09	13.04	9.58	113.2
Glutamic acid	13.46	11.81	7.72	91.6
Ammonia*	1.32	1.24	6.53	77.7
Total	113.70	98.21	99.76	934.3

* The values of serine and threonine were corrected by 10 per cent and 5 per cent, respectively, for the destruction during acid hydrolysis of protein, and the value of ammonia was correspondingly reduced according to Rees (*14*).

amylase A (*3*) and bacterial amylase of *B. subtilis N*, are given in Table IV. Certain similarities are perceptible among them, that is, aspartic acid contents are relatively high and methionine contents are low. The results suggest that the structural responsibility for the enzymatic action might be attributed to some particular peptide part having specific amino acid sequences, but not to their amino acid compositions. The determination of the amino and carboxyl terminal residues is in progress by using Sanger's DNP method and hydrazinolysis method (*13*), respectively, and the results will be published in the succeeding paper (*12*).

TABLE IV

The Comparison of Amino Acid Composition of Amylases

Amino acids \ Amylases	g. of amino acid per 100 g protein			
	Pancreatic amylase from swine (1)	Human salivary amylase (2)	Taka-amylase A (3)	Bacterial amylase of *B. subtilis* N
Glycine	6.7	6.82	6.59	5.64
Alanine	6.9	4.43	6.80	6.02
Valine	7.8	6.89	4.69	5.55
Leucine }	11.5	5.77	8.30	6.42
Isoleucine }		5.80	5.20	3.97
Proline	3.6	3.6	4.18	4.14
Phenylalanine	10.1	7.20	4.25	5.85
Tyrosine	5.3	5.51	9.55	8.31
Tryptophan	6.7	7.2	3.97	6.19
Serine	4.1	7.8	6.48	6.24
Threonine	3.9	4.5	10.86	6.36
Cystine	2.3	4.4*	1.60	0
Methionine	2.1	2.4	2.20	1.26
Arginine	5.8	8.75	2.72	6.78
Histidine	3.9	3.24	2.02	3.80
Lysine	4.9	6.33	5.94	7.30
Aspartic acid	14.5	19.3	16.53	15.09
Glutamic acid	10.5	9.6	6.95	13.46
Ammonia	1.6	—	1.50	1.32

* On the assumption that total sulfur minus methionine sulfur is cystine sulfur.

SUMMARY

The amino acid compositions of crystalline bacterial amylase of *B. subtilis N* has been determined by chromatographic analyses.

The authors wish to express their sincere thanks to Mr. Bunji Hagihara for his kind supply of bacterial amylase.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

THE DECOMPOSITION OF β-MERCAPTOPYRUVIC ACID BY ESCHERICHIA COLI AND ITS EXTRACTS

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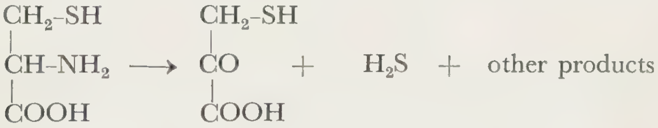
(Received for Publication, July 26, 1956)

Fromageot *et al.* (*1*, *7*) proposed a scheme for the decomposition of cysteine involving the enzymatic desulfhydration followed by spontaneous deamination. Recently Suda (*2*, *3*) Hanson and Mantel (*4*) and Tamiya (*5*) reported that in experiments with certain bacteria, the liberation of hydrogen sulphide from cysteine was preceded by the disappearance of cysteine and by liberation of ammonia ; the reaction in these cases, therefore, essentially different from Fromageot's scheme of cysteine desulfhydrase.

One of the authors proposed the following reaction scheme in the previous paper (*6*) ;



$$\begin{array}{l} CH_2\text{-}SH \\ | \\ CH\text{-}NH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2\text{-}SH \\ | \\ CO \\ | \\ COOH \end{array} + \quad H_2S \quad + \text{ other products}$$

A derivative of thiol keto acid was isolated from a reaction mixture in which liberation of hydrogen sulphide was inhibited by the addition of arsenite (*5*).

Meister reported that the enzymic decomposition of β-mercaptopyruvic acid was catalyzed by an enzyme from mammaliam liver (*8*). Garreau (*13*) and Hanson *et al.* (*4*) reported that this substance is decomposed also by suspensions of *Escherichia coli.* The present paper deals with the decomposition of β-mercaptopyruvic acid by cell suspensions and partially purified enzyme preparations from *Escherichia coli.*

EXPERIMENTAL

Cell Preparations—*Escherichia coli* strain Taki-2 was used throughout the experiments.

After cultivation at 37° on a normal agar slant for 18 hours or in a liquid medium under shaking at 30° for 12–15 hours (4 per cent peptone, 4 per cent meat extract pH 7.0), the cells of *E. coli* were collected and washed with water three times by centrigugation. In experiments carried out with intact cells, suspensions of washed cells in $M/15$ phosphate buffer at pH 7.0 were used. In some of the other experiments, freeze-dried cells were used; dried cells kept in vacuo in a refrigerator did not change their activity for several months.

Enzyme Preparations—Two g. of freeze-dried cells and 20 g. of alumina powder were kneaded with $M/15$ potassium dihydrogen phosphate, adjusted to pH 8.0 with N sodium hydroxide and ground in a machinery mill for 4 hours at room temperature. Then the mixture was adjusted to pH about 7.0 by the addition of 20 ml. of $M/15$ potassium dihydrogen phosphate and centrifuged at 4000 r.p.m. for 10 minutes. The supernatant was discarded; most of the enzyme protein was adsorbed on the alumina. The enzyme was extracted from the alumina precipitate by adding 50 ml. of $M/15$ sodium monohydrogen phosphate. After centrifugation at 16,000 r.p.m. for 10 minutes, a clear light yellow supernatant was obtained, which was dialyzed against flowing water overnight. The dialysate gave a white powder on freeze-drying. The enzyme prepation could be kept unchanged for several months and used as partially purified enzyme.

Conditions for the Reaction—Unless otherwise stated, the conditions for the reaction were as follows: All the reaction components were dissolved in $M/15$ phosphate buffer, pH 7.0. The reaction was carried out at 37° under continuous bubbling of air or nitrogen gas. This procedure insured not only aerobic or anaerobic conditions of the reaction medium, but also continuous removal of hydrogen sulphide produced by the reaction.

Determination of Hydrogen Sulphide—Hydrogen sulphide formation was determined by Szent Lorant's method (*9*). The hydrogen sulphide was passed with a stream of air into a zinc acetate solution. Upon the addition of an acid solution of iron alum and dimethyl-*p*-phenylene diamine, the sulphur of the zinc sulphide was incorporated into methylene blue molecule which was synthesized by this procedure. The colour developed was measured electrophotometrically at 610 mμ.

Determination of Free Sulphur—Free sulphur was estimated essentially by the method described by Smythe (*14*). Free sulphur was reduced to hydrogen sulphide by the addition of thioglycolic acid, and subjected to colorimetric analysis as described above.

Determination of Pyruvic Acid—One ml. of 10 per cent mercuirc sulphate solution in 3.6 N sulphuric acid was added to one ml. of reaction mixture. Thiol compounds and protein were precipitated by this procedure. After centrifugation, pyruvic acid content in the supernatant was determined by the method of Friedemann and Haugen (*10*).

Determination of Other Compounds—Carbon dioxide was determined by Warburg's manometric method. Volatile organic acids were measured titrimetrically after distillation of the reaction mixture in microacetyl determinating apparatus.

Chemicals—Ammonium β-mercaptopyruvic acid was prepared as described by

Parrod (*11, 12*). The ammonium salt was used throughout these experiments. The results obtained with ammonium salt were similar to those obtained with sodium salt, which was prepared by passing a saturated aqueous solution of ammonium salt through a Amberlite IRC-120(Na-form)column.

RESULTS

Fundamental Conditions for the Decomposition of β-Mercaptopyruvic Acid by Cell Suspensions of Escherichia coli—Some preliminary experiments were carried out to find out the optimal conditions for the reaction. In these experiments only hydrogen sulphide production was followed. In none of the experiments, hydrogen sulphide was produced in the absence of the substrate.

β-Mercaptopyruvic acid is fairly unstable in aqueous solution and decomposes slowly producing a small amount of hydrogen sulphide. However, with added *E. coli* suspension, the decomposition of β-mercaptopyruvic acid was greatly stimulated. The rate of hydrogen sulphide production from β-mercaptopyruvic acid was much more rapid than that from cysteine under similar conditions. (Fig. 1)
With cell suspensions, the production of hydrogen sulphide from β-mercaptopyruvic acid was accompanied by the production of carbon

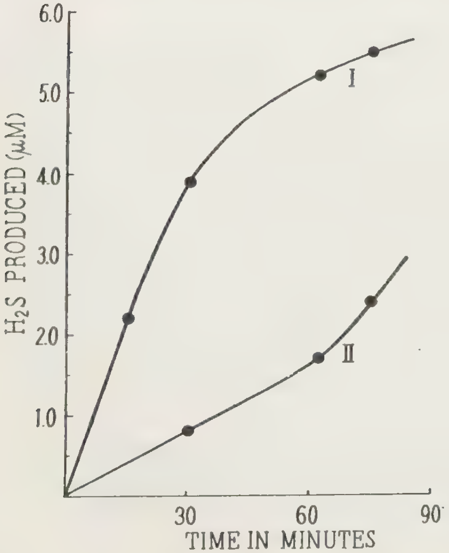


Fig. 1. Hydrogen sulphide production from β-mercaptopyruvic acid and cysteine.

Each reaction tube contained 20 μ moles of substrate (I: β-mercaptopyruvic acid, II: cysteine), 40 mg. of lyophylized cells of Escherichia coli, and phosphate buffer, pH 7.0 (final *M*/15) in total five ml. Reaction temperature 37°. Gas phase: air.

dioxide. The hydrogen sulphide production did not proceed more than 50–60 percent of the amount calculated from substrate concentration. With cell suspensions there was no accumulation of free sulphur, as was observed in experiments with partially purified enzyme.

Effect of Addition of Various Substances on the Enzymic Decomposition of β-Mercaptopyruvic Acid—In the previous paper (*6*), one of the authors reported that the decomposition of cysteine by *Escherichia coli* suspensions was markedly stimulated by the addition of various respiratory substrates such as glucose or organic acids. We therefore studied the stimulating effect of these substances on the decomposition of β-mercaptopyruvic acid. The addition of glucose had no effect on the reaction rate. Potassium cyanide, which showed inhibiting effect on the formation of both ammonia and hydrogen sulphide from cysteine in the presence of *Escherichia coli* suspension, exhibited 50 percent inhibition at the concentration of 5.4×10^{-3} *M*. Arsenite, which showed an inhibitory effect on the formation of hydrogen sulphide from cysteine without affecting ammonia production from it, showed a strong inhibitory effect on the formation of hydrogen sulphide from β-mercaptopyruvic acid.

Effect of Thioglycolic Acid on the Reaction—The addition of thioglycolic acid increased the yield of hydrogen sulphide from β-mercaptopyruvic acid by *Escherichia coli* suspensions (see Fig. 2), but it inhibited the carbon dioxide evolution at the same time. (Fig. 3)

As described below, one of the primary decomposition products from β-mercaptopyruvic acid is pyruvic acid. Thioglycolic acid, therefore, seems to inhibit further decomposition of pyruvic acid and thus inhibits the carbon dioxide evolution (Fig. 4).

The Decomposition of β-Mercaptopyruvic Acid by Partially Purified Enzyme Preparation—Enzyme preparations made as described in experimental part showed a high activity in β-mercaptopyruvic acid decomposition. In this case also, the hydrogen sulphide or pyruvic acid formation in the presence of thioglycolic acid did not proceed more than 45–50 per cent of added substrate. These preparations had no cysteine desulfhydrase activity. The activity of the crude preparation, *i.e.*, the supernatant of alumina ground material, remained unchanged after heating in a boiling water bath for 10 minutes.

The enzyme was decomposed more rapidly by heating after further purification by means of adsorbtion and elution on alumina and fractionation with acetone or ammonium sulphate. The enzyme activity was also destroyed by acid or pepsin hydrolysis (Fig. 5).

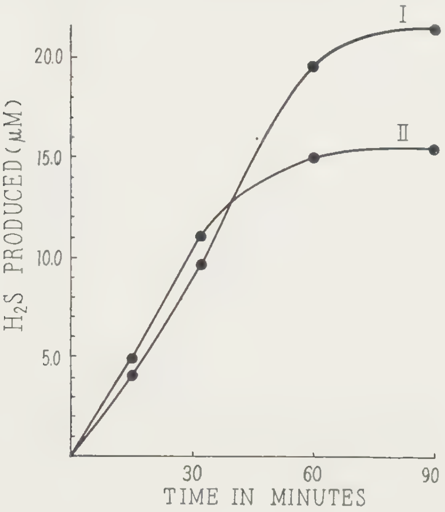


FIG. 2. Effect of the addition of thioglycolic acid Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 20 mg. of lyophylized cells, and phosphate buffer, pH 7.0 (final *M*/15). To tube I, 40 μ moles of thioglycolic acid was added.

Total volume: 5 ml. Temperature: 37°. Gas phase: air.

Further Purification of the Enzyme—The enzyme prepared as described in the experimental part was subjected to further purification by ammonium sulphate fractionation. Dried enzyme preparation was dissolved in 10 volumes of water and fractionated by the addition of solid ammonium sulphate. The activity was concentrated in a fraction of 0.4–0.8 ammonium sulphate saturation.

Effect of Oxygen upon the Reaction—The enzymatic decomposition of β-mercaptopyruvic acid proceeded both in the presence and absence of oxygen. However, in experiments with cell suspension, the reaction velocity was greater when air was aspirated than when nitrogen was used. In an experiment with partially purified enzyme, the reaction rate was almost equal under both conditions (Figs. 6 and 7).

Identification of Reaction Products—Pyruvic acid: 500 mg. of the partially purified enzyme and 2 millimoles of β-mercaptopyruvic acid were dissolved in 150 ml. of *M*/15 phosphate buffer (pH 7.0). After two hours' incubation, under continuous aspiration of nitrogen gas at 37°, an equal volume of 10 per cent mercuric sulphate in 3.6 *N* sulphuric

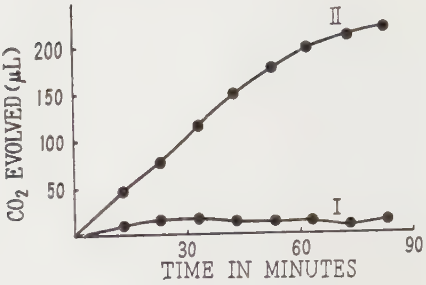


FIG. 3. The effect of thioglycolic acid addition on the evolution of carbon dioxide.

Each Warburg vessel contained: in the main compartment, 20 μ moles of mercaptopyruvic acid, and phosphate buffer pH 7.0 (final $M/15$) in a total amount of 2.5 ml., to tube I 20 μ moles of thioglycolic acid was added; in the side arm, 0.5 ml. of lyophylized cell suspension (40 mg/ml), in the center well, 0.2 ml. of lead acetate (2 M). The reaction was initiated by the addition of the content of side arm.

Temperature: 37°. Gas phase: air.

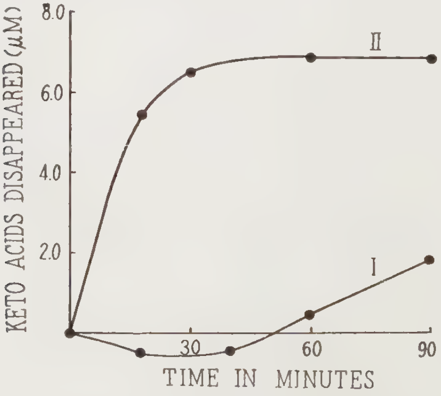


FIG. 4. The effect of addition of thioglycolic acid on the disappearance of keto acids.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 20 mg. of lyophylized cells and phosphate buffer pH 7.0 (final $M/15$) in a total amount of 5 ml. to tube I, 40 μ moles of thioglycolic acid was added.

Temperature: 37°. Gas phase: air.

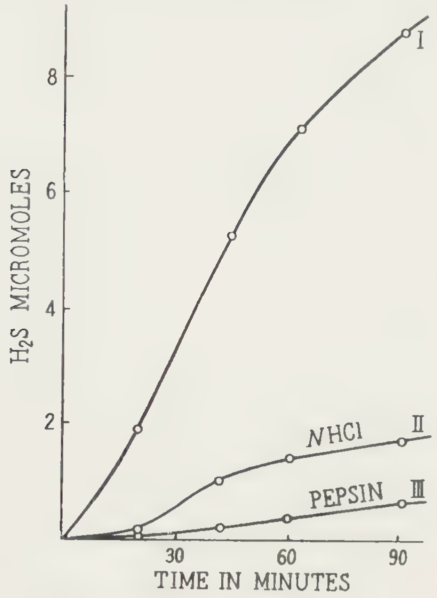


Fig. 5. Inactivation of Enzyme by hydrolysis and digestion.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 40 μ moles of thioglycolic acid, 1 ml. of enzyme solution (43 mg. acetone powder of purified enzyme by acetone fractionation) or hydrolysed material, and phosphate buffer pH 7.0 (final *M*/15) in a total amount of 5 ml.

Temperature 37°. Gas phase: air.

The hydrolysed or digested material was prepared as follows: 43 mg. of enzyme preparation was dissolved in 2 ml. of distilled water.

N·HCl: 1 ml. of 2 *N* HCl was added to 1 ml. of this enzyme solution. It was boiled on boiling water for 10 minutes. After cooling it was used as hydrolysed material.

Pepsin: 1 ml. of 0.12 *N* HCl was added to 1 ml. of the enzyme solution and 5 mg of crystal pepsin (2 times recrystalized from alcohol) was added.

After incubation for 1 hour, this reaction mixture was used as pepsin digest.

I: Control (enzyme without treatment).
II: *N* HCl (*N* HCl hydrolysate in place of enzyme).
III: Pepsin (pepsin digest in place of enzyme).

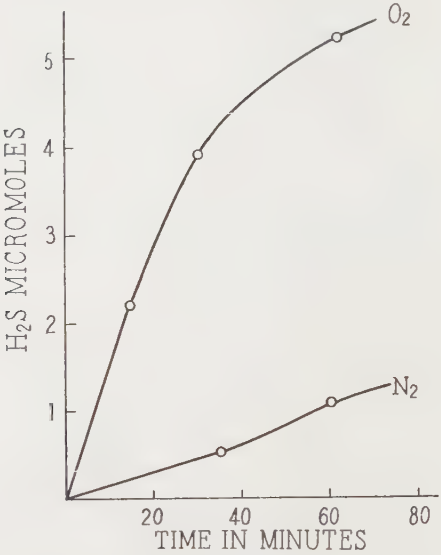


FIG. 6. Effect of presence of oxygen on hydrogen sulphide production by lyophilized cells.

Each reaction tube contained 20 μ moles of mercaptopyruvic acid, 1 ml. of cell suspension (1.03 mg./ml.), and phosphate buffer pH 7.0 (M/15) in a total amount of 5 ml.

Temperature: 37°. Gas phase: air or nitrogen gas.

acid was added to remove protein and unreacted substrate (Meister *et al.* (*8*)). The yellow precipitate was filtered off and concentrated hydrochloric acid was added to the filtrate to bring the final concentration up to 2 N. 80 ml. of 0.5 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid was then added. After standing for 20 minutes at room temperature, yellow precipitate of hydrazone was obtained. The hydrazone was extracted into ethyl acetate together with excess hydrazine, and dried *in vacuo*. The orange yellow material obtained here was dissolved in a small amount of ethyl acetate and added to a celite column, buffered at pH 6.8. Ether was passed down the column and the material separated into three bands. A band which showed the R value of 0.3–0.4 was extracted in sodium bicarbonate solution, re-extracted into ether and treated with calcium chloride and dried in vacuo. 2,4-dinitrophenylhydrazone obtained here was identified as that of pyruvic acid

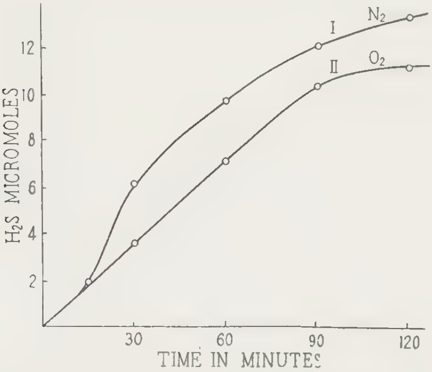


FIG. 7. Effect of presence of oxygen on hydrogen sulphide production by enzyme preparation.

Each reaction tube contained 40 μ moles of mercaptopyruvic acid, 40 μ moles of thioglycolic acid, 1 ml. of dialysed enzyme preparation, and phosphate buffer pH 7.0 (*M*/15) in a total amount of 5 ml. Temperature: 37°. Gas phase: I: nitrogen gas. II: air.

by measurement of mixed melting point.

hydrazone isolated m.p.=217°
pyruvic acid 2,4-dinitrophenylhydrazone m.p.=221°
mix test m.p.=219°

Sulphur: Meister *et al.* reported that a decomposition product of β-mercaptopyruvic acid in the presence of a mammalian liver enzyme preparation was sulphur. In experiments with partially purified enzyme from *Escherichia coli*, we were able to show similar fact, *i.e.*, the formation of free sulphur. In the experiments with cell suspensions, however, this substance was not detected. This is expected because in living cells sulphur is readily reduced to hydrogen sulphide.

The decomposition of β-mercaptopyruvic acid by partially purified enzyme was accompanied by increase in turbidity of the reaction medium, although considerable evolution of hydrogen sulphide took place at the same time. This precipitate was treated by essentially the same method as described by Meister *et al.* (*8*). The amount of sulphur in the precipitate was estimated by the addition of thioglycolic acid, followed by St. Lorant's colorimetric method.

These results showed that the decomposition products from β-mercaptopyruvic acid were pyruvic acid, free sulphur and hydrogen sulphide. The molar ratio of formation of free sulphur and hydrogen sulphide was variable but the total amount of these two substances showed a stoichiometric relation to pyruvic acid production (Table I).

TABLE I

Experiment No.	Formation of pyruvic acid	Evolution of hydrogen sulphide	Formation of free sulphur	H_2S+S
	μ moles	*μ moles*	*μ moles*	*μ moles*
1	8.32	1.78	6.50	8.28
2	7.27	3.69	3.35	7.04
3	2.73	1.64	0.96	2.60

Formation of pyruvic acid, hydrogen sulphide and free sulphur. Each reaction tube contained: 20 μ moles of β-mercaptopyruvic acid, 1 ml. of enzyme preparation and phosphate buffer (pH 7.0) in total 5 ml. Temperature: 37° Gas phase: air. The figure in the table are expressed in μ moles of substances formed in 80 minutes.

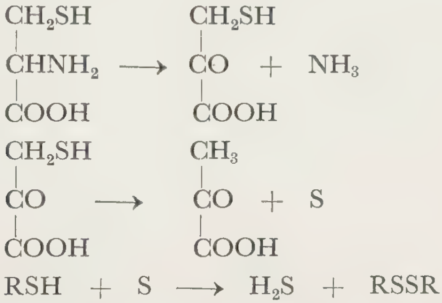
The stimulating effect of hydrogen sulphide production by the addition of thioglycolic acid can be explained as being due to the reduction of free sulphur by this substance. In the presence of this substance, equimolecular amount of hydrogen sulphide and pyruvic acid were formed.

Other Porducts: The decomposition of β-mercaptopyruvic acid into pyruvic acid and free sulphur did not proceed more than 45–50 per cent under our experimental conditions. This was not due to the inactivation of enzyme because the second addition of freshly prepared enzyme solution did not effect further production of hydrogen sulphide or pyruvic acid. It is clear, therefore, that β-mercaptopyruvic acid was converted into some inactive substance or substances (*8*). Among the products which were tested for only glyoxylic acid was detected. which corresponded to two percent of added β-mercaptopyruvic acid.

Formaldehyde, Acetaldehyde, other keto-compounds, formic acid, and acetic acid were not detected. Fresh β-mercaptopyruvic acid was kept at room temperature for 7–8 hours or aerated for 30 minutes, decomposed and the keto-group of the acid can no longer be detected. With such a solution, no hydrogen sulphide production was observed upon the addition of enzyme preparation together with thioglycolic acid. The low yield of hydrogen sulphide from the enzymic reaction may thus be due to this non-enzymic decomposition.

DISCUSSION

In the previous paper (*6*) one of the authors proposed a scheme of cysteine decomposition by *E. coli* suspensions which involved the intermediary formation of β-mercaptopyruvic acid. The results presented in this paper shows that this acid is further decomposed into pyruvic acid and free sulphur, a part of which is reduced to hydrogen sulphide. In the presence of added thiol compound or with intact cell suspensions, all of the sulphur is reduced to hydrogen sulphide, while in the absence of it only a part of sulphur is reduced to hydrogen sulphide by thiol group of substrate itself. Now we can visualize the whole scheme of cysteine decomposition by *Escherichia coli* under aerobic conditions as follows:



$$(1)\quad \begin{array}{l} CH_2SH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_2SH \\ | \\ CO \\ | \\ COOH \end{array} + NH_3$$

$$(2)\quad \begin{array}{l} CH_2SH \\ | \\ CO \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} + S$$

$$(3)\quad RSH + S \longrightarrow H_2S + RSSR$$

The first step, which was studied in the previous paper, requires the presence of oxygen or nitrate and is inhibited by potassium cyanide. The second step is specifically inhibited by arsenite. With intact cell suspensions in the presence of thiol compounds, the free sulphur produced by the second reaction is reduced to hydrogen sulphide.

SUMMARY

1. β-Mercaptopyruvic acid is decomposed by an enzyme prepared from *E. coli*.

2. Products of this reaction are pyruvic acid and elemental sulfur. But in the case of reaction with intact cell β-mercaptopyruvic acid is further decomposed to carbon dioxide and hydrogen sulfide.

3. This enzyme is stable against heat in crude state. However, in

pure state this thermal stability is lost.

4. The probability of this compound to be in the metabolic path of cysteine is discussed.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

INTERRELATION BETWEEN THE FUNCTION OF HEMEPROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

IX. PERTURBATION OF CYTOCHROME C

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(Received for publication, July 29, 1956)

Sodium salt of salicylate, benzoate and various fatty acids, when added to hemoglobin or myoglobin solution, the latter molecule undergoing reversible denaturation (or perturbation in the meaning of Holden) (*1*, *2*, *3*). According to the grade of this protein modification of hemoglobin as well as myoglobin, the intrinsic activity of oxidase which has so far been remaining latent in the native molecule becomes then active as reported by Kikuchi and Tomimura (*4*). In the more progressed state of the modification, these hemeproteins show the absorption spectra of hemochrome or hemichrome as reported by Tsushima (*1*). Also, catalase, when similarly modified in its protein part, its essential enzymatic activity was variously modified as reported by Suzuki, Tomimura and Mizutani (*5*).

From their interest on the relation between the structural modification of hemeproteins and the function of them, the present authors intended to study the modification of enzymatic function of cytochrome c in the presence of various perturbators. Cytochrome c is known to be much more resistent against acid, alkali and heat denaturation than other hemeproteins.

EXPERIMENTAL

Cytochrome c was prepared from beef heart by the method of Keilin and Hartree (*6*). As the perturbators, sodium benzoate, sodium salicylate and sodium lauryl sulfate in pure crystalls were used dissolved in distilled water. The pH of the solutions were adjusted to 7.4.

For the measurement of the optical density, the electric spectrophotometer of Hitachi Co. Model EPB-V was used. The oxidase activity was investigated manometrically by the Warburg techniqe as described below.

The Spectral Observations—A definite volume of cytochrome c solution was pipetted into a small beaker of 20 ml content and a definite volume of the solutions of perturbators in varying concentrations was added. The pH of the solution was held at 7.4 with 0.1 *M* phosphate buffer. The total volume of the mixed solution was made up to 3.0–4.0 ml. After keeping 30 minutes at room temperature, a small amount of powdered dithionite was added to the solution and the absorption figure was then determined.

For the determination of the CO-binding ability of the modified cytochrome c, the reaction solution was pipetted into the cuvette, reduced by dithionite, covered with liquid paraffine and after being bubbled with CO-gas for 2–3 minutes, the changes of the absorption figure were measured.

Determination of the Oxidase Activity—Cytochrome c solution, 0.1 *M* phosphate buffer (pH 7.4) and the solution of perturbator were mixed and placed in the main chamber and the neutralized ascorbate was placed in the side arm as the substrate. After the vessels were shaken for 15 minutes at 37°, the solutions were mixed and the rate of oxygen uptake were then estimated.

RESULTS

I. Spectroscopical Observations

On the addition of above mentioned perturbators to hemoglobin and methemoglobin solution, absorption figures corresponding to hemochrome and hemichrome can be obtained, respectively. These figures indicate the existence of a bonding of heme-Fe to another globin-N which became accessible to the bonding through an unfolding of the molecule of protein portion of these hemeproteins as the result at their perturbation. In the case of the heme-Fe of cytochrome c, its 5th and 6th coordination are saturated with its globin-N, delivering thus the hemochrome or hemichrome structure (*7*). Further, the hydroxyethyl side chains of its heme portion are forming thioether bonding by combination with its globin-SH (*8*).

Some alteration in these bondings of heme-Fe with protein portion of the cytochrome c may well be expected when its protein moiety is structurally modified by the action of perturbators. To investigate on this point, following spectroscopical observations were made.

Experiment with Sodium Salicylate—When, under the condition described in Fig. 1., sodium benzoate at the varying concentrations in the range of 0.5 to 2.0 *M* was added to the cytochrome solution, the optical density at 550 mμ was decreased while those at 560–600 mμ and 450–500 mμ as well as that at about 535 mμ were increased. But the absorption spectrum as a whole has not essentially altered as indicated in Fig. 1.

Experiment with Sodium Salicylate—In the similar experiment with

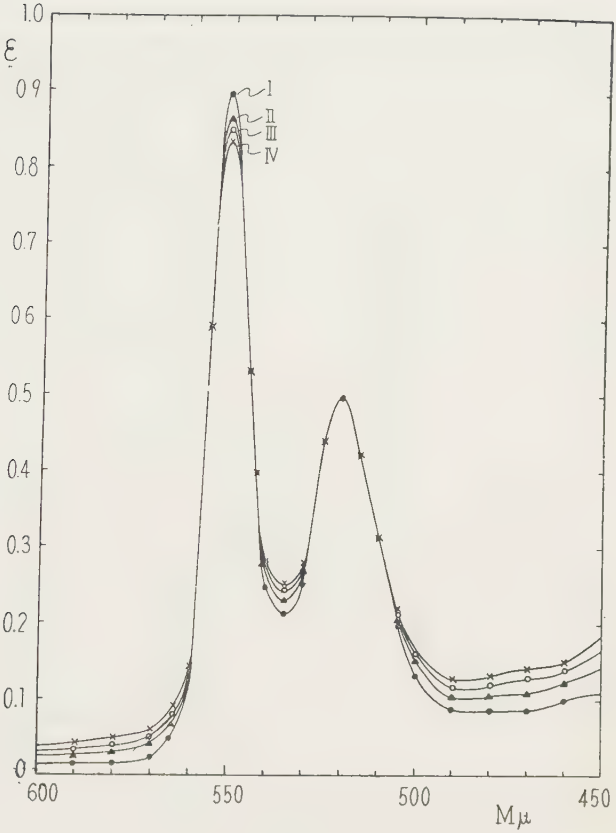

FIG. 1. The absorption figures obtained by addition of benzoate in varied concentrations.

Concentration of cytochrome c: 3.2×10^{-5} *M.*

Concentrations of benzoate: I, 0.0–0.8 *M.*; II, 1.6 *M.*; III, 1.76 *M.*; IV, 1.84–2.0 *M.* pH: 7.4 (0.1 *M* phosphate buffer).

sodium slaicylate, when salicylate was added in the concentration above 1.7 *M*, the absorption maximum at 550 mμ was gradually decreased and the maximum has shifted slightly toward longer wave length side. At the concentration of salicylate above 3.5 *M*, the absorption at 550 mμ was significantly decreased. On higher salicylate concentration, the absorption spectrum may cause a further alteration. But it was not

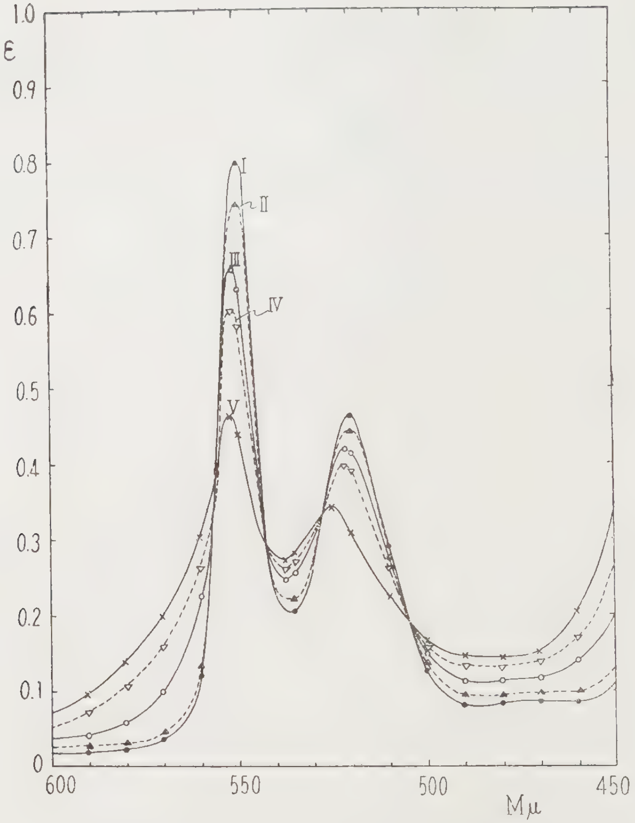

Fig. 2. The absorption figures obtained by addition of salicylate in varied concentrations.

Concentration of cytochrome c: 3.0×10^{-5} *M*.

Concentrations of salicylate: I, 0.0–1.3 *M*; II, 1.7 *M*; III, 2.0 *M*; IV, 2.5 *M*; V, 3.5 *M*.

pH: 7.4 (0.1 *M* phosphate buffer).

possible to examine with more concentrated salicylate. During the course of these absorption changes, no clearcut isosbestic point was demonstrable. In the case of the similar experiment with lauryl sulfate as described followingly, however, isosbestic points could clearly be demonstrated at 556, 540, 531, and 503 mμ during the course of the absorption changes. This discrepancy of the results between salicylate and lauryl sulfate may be accounted for the solvent effect due to the high concentration of salicylate required for the absorption change.

Experiment with Sodium Lauryl Sulfate—Under the condition described in Fig. 3, varying concentrations of lauryl sulfate were added to the cytochrome solution. During the course of the resulting absorption change with increasing lauryl sulfate concentration, isosbestic points were clearly demonstrated at 556, 540, 531, and 503 mμ. Lauryl sulfate

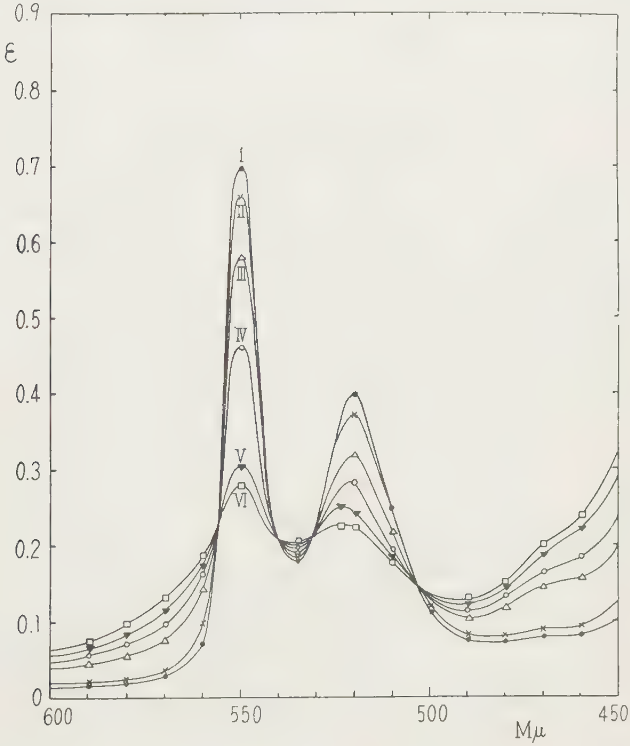

Fig. 3. The absorption figures obtained by addition of lauryl sulfate in varied concentrations.

Concentration of cytochrome c: 2.5×10^{-5} *M*.

Concentrations of lauryl sulfate: I, 0.0 *M*; II, 5×10^{-4} *M*; III, 1×10^{-3} *M*; IV, 2×10^{-3} *M*; V, 3×10^{-3} *M*; VI, 4×10^{-3} *M*. pH: 7.4 (0.1 *M* phosphate buffer).

was effective for the absorption change of reduced cytochrome c in much lower concentration range than benzoate or salicylate. This relation is similar to that for hemoglobin or myoglobin. The Curve VI in Fig. 3 is the final spectrum of cytochrome c effected by lauryl sulfate. The absorption maxima in the final state were recorded at 550 and 525 mμ, the former being stronger than the latter.

Irreversibility of the Absorption Change—The reversibility of the resulting absorption change was then investigated. It was found that dilution of the perturbators as well as dialysis was not effective to retain the initial absorption spectra of cytochrome c. Thus, the structural modification of the protein portion of cytochrome c caused by these perturbators may be assumed to be irreversible.

II. The Increase of CO-Binding Affinity of Cytochrome c According to Its Protein Modification

The reduced cytochrome c in its native state is known to be incapable of forming CO-compound. When, however, a denaturating change has occurred in its protein portion, it is known to combine with carbon monoxide. Tsuo (*9*), for example, followed up the digestion grade of cytochrome c by trypsin and pepsin using its CO-combining affinity as an indication. The present authors investigated thus the protein modification of cytochrome c in the present experiment by measuring its CO-binding affinity as a criterion.

Experiment with Benzoate—Under the condition described in Fig. 4, sodium benzoate was added in varying concentrations to cytochrome c solution, reduced by dithionite followed by the saturation with CO-gas and the absorption was finally determined. As indicated in Fig. 4 the absorption spectrum of cytochrome c was changed in accord with increasing concentrations of benzoate, isosbestic points being clearly demonstrated at 555, 542, 527, and 505 mμ. The Curve V in Fig. 4 is the final absorption spectrum of the cytochrome c in its modified state. This figure was found to be identical to the figure obtained by Tsuo (*10*) in the CO-compound of pepsin modified cytochrome c. Assuming this curve V as the final figure of the CO-compound of the modified cytochrome c, the percentages of the formation of CO-compound were calculatively obtained by use of a nomogram obtainable from the absorption change indicated in Fig. 4. These percentages were then plotted against log concentration of benzoate added. The plots make a sigmoid curve of a high order reaction as shown by curve I in Fig. 5.

From the experimental results above described, it was plausibly

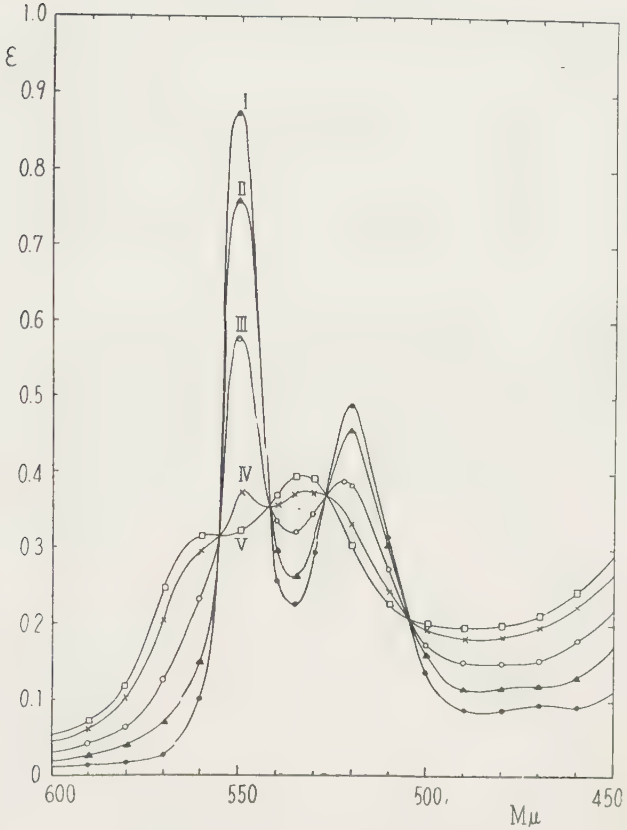

FIG. 4. The absorption change of ferrocytochrome c by addition of varying benzoate concentrations on saturation with carbon monoxide.

Concentration of cytochrome c: 3.2×10^{-5} *M*.

Concentrations of benzoate: I, 0.0–0.8 *M*; II, 1.6 *M*; III, 1.76 *M*; IV, 1.84 *M*; V, 1.92–2.0 *M*.

pH: 7.4 (0.1 *M* phosphate buffer).

assumed that cytochrome c was affected by benzoate in its protein portion to a denaturative change which makes it possible to combine with carbon monoxide. It was then investigated whether this structural change of cytochrome c protein is reversible. The reversibility was evidenced by a dilution experiment as follows. A cytochrome c solution which had preliminary been mixed with benzoate solution of a efficiently high concentration to bring about the final absorption spectra of CO-compound and kept on to stand for a short period, was diluted with water until the final benzoate concentration was lowered to less than 0.8 *M*. To this solution, dithionite was added and CO-gas was introduced. On this procedure, the absorption spectrum of CO-compound was vanished and those of the native cytochrome c could be retained. It was assumed thus that this modified state of cytochrome c protein which allows to form a CO-compound is of a completely reversible one.

Experiment with Salicylate—A similar series of experiments was conducted by applying sodium salicylate instead of benzoate. The results obtained were fairly analogous to those of the experiment with benzoate (Fig. 6). When the percentages of the formation of CO-compound in this case was plotted against log concentration of salicylate added, a sigmoid curve was obtained as shown by Curve II in Fig. 5.

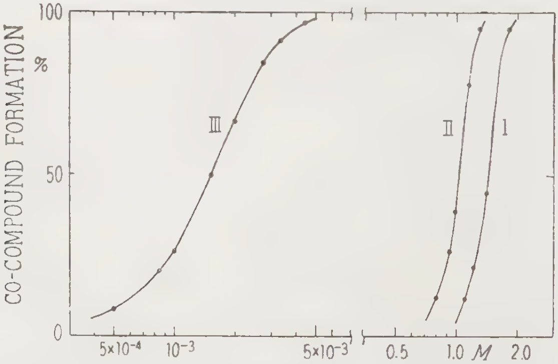

Fig. 5. Plots of CO-compound formation vs log concentration of perturbators.

Curves I, II, and III were obtained by addition of benzoate, salicylate, and lauryl sulfate, respectively.

Comparing Curve II with Curve I in Fig. 5, it is evident that for the perturbation of cytochrome c protein, the effective concentration of salicylate is much lower than that of benzoate. And the modification of cytochrome c protein is reversible in both cases of perturbation which could be evidenced by dilution experiment.

Experiment with Lauryl Sulfate—In the *Experiment I*, it was proved

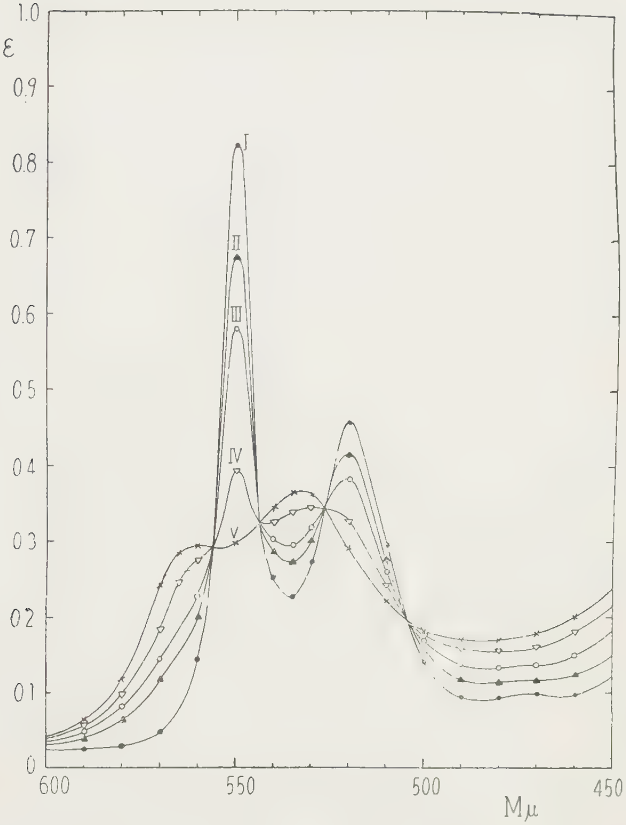

Fig. 6. The absorption change of ferrocytochrome c by addition of salicylate on saturation with carbon monoxide.

Concentration of cytochrome c: $3.0 \times 10^{-5} M$.

Concentrations of salicylate: I, $0.0 M$; II, $0.8 M$; III, $1.0 M$; IV, $1.1 M$; V, $1.3 M$.

pH: 7.4 ($0.1 M$ phosphate buffer).

that the absorption change of reduced cytochrome c was caused by lauryl sulfate in much lower concentration than benzoate or salicylate. The formation of CO-compound of cytochrome c being affected by lauryl sulfate was then investigated.

Under the experimental condition indicated in Fig. 7, lauryl sulfate was added to the solution of cytochrome c, reduced with dithionite and

CO-gas was introduced finally. Then the absorption spectrum of the solution was gradually changed step by step toward the final figure of CO-compound with the increasing concentrations of lauryl sulfate added. During these absorption changes, isosbestic points were clearly demonstrated at 557, 542, 528, and 505 mμ, so the percentages of the CO-compound formed was calculatively obtained from the absorption change in Fig. 7. These percentages were plotted against log concentration of lauryl sulfate. A sigmoid curve indicated in curve III of Fig. 5 was then obtained. As seen from this figure, lauryl sulfate was effective for the protein modification of this cytochrome c at the much lower concentration than benzoate or salicylate.

III. The Oxidase Activity of the Modified Cytochrome c

From the spectroscopical observations above described, it was assumed that the protein portion of cytochrome c was structurally modified by the effect of benzoate or salicylate added and that the resulted functional modification of heme-Fe (for the bonding of carbon monoxide and of protein-N) through these perturbators may be accounted for this structural modification. Then, if the spectroscopically evidenced functional modification of heme is actually caused by the addition of these perturbators, it will well be expected that the intrinsic oxidase activity of its heme, which has so far been remaining latent in the native state of the molecule, may be brought to restoration. Cytochrome c is non-autoxidizable in the native state ; this is restrictively controlled by the chemical properties of its protein portion, and when once the protein portion is structurally modified definitely, it becomes then autoxidaizable.

In order to evidence the catalytic change of cytochrome c by the addition of various perturbators mentioned above, the present authors intended to study the increment of the oxidase activity of the modified cytochrome c after the treatment with these perturbators by use of ascorbic acid as the substrate.

Under the condition indicated in Fig. 8, the oxidation rate of ascorbate by the modified cytochrome c was investigated manometrically from the rate of oxygen uptake. The rate was increased with increasing benzoate concentrations added to the cytochrome c solution so far as the final benzoate concentration reached up to 1.0 *M*. Above 1.0 *M* of benzoate, however, this promoting effect of benzoate was rather weakend. Then, the increment of oxygen uptake was calculated in percentage and this was plotted against the log concentration of benzoate. The plot was shown by Curve I in Fig. 9. As indicated

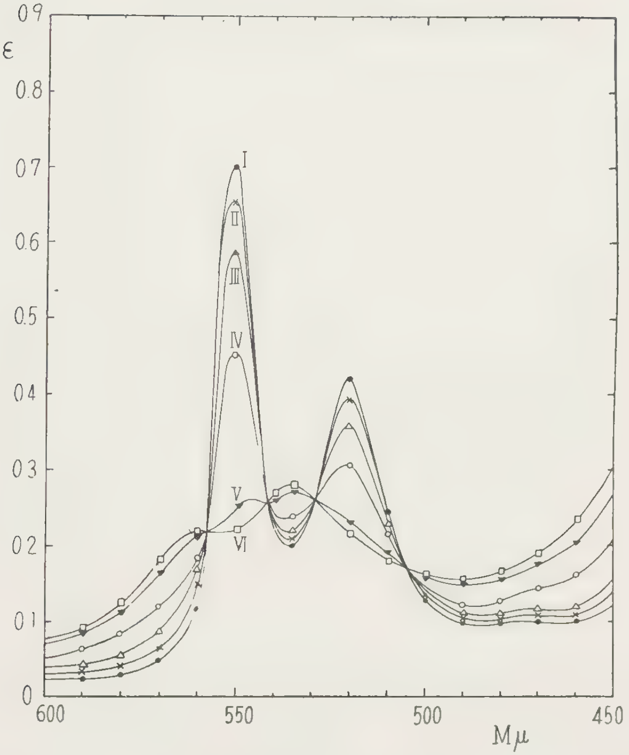

FIG. 7. The absorption change of ferrocytochrome c by addition of lauryl sulfate on saturation with carbon monoxide.
Concentration of cytochrome c: 2.5×10^{-5} *M*.
Concentrations of lauryl sulfate: I, 0.0 *M*; II, 5×10^{-4} *M*; III, 1×10^{-3} *M*; IV, 2×10^{-3} *M*; V, 3×10^{-3} *M*; VI, 4×10^{-3} *M*.
pH: 7.4 (0.1 *M* phosphate buffer).

by this curve, the increment reached maximum at about 1.0 *M* of benzoate and above this concentration the promoting effect of benzoate was decreased.

Comparing Curve I in Fig. 5 (the formation of CO-compound) with Curve I in Fig. 9 (increment of oxidase activity), it was indicated that the promotion of oxidase activity was attained by far lower concentration range of benzoate added than that for the formation of CO-compound of thus modified cytochrome c.

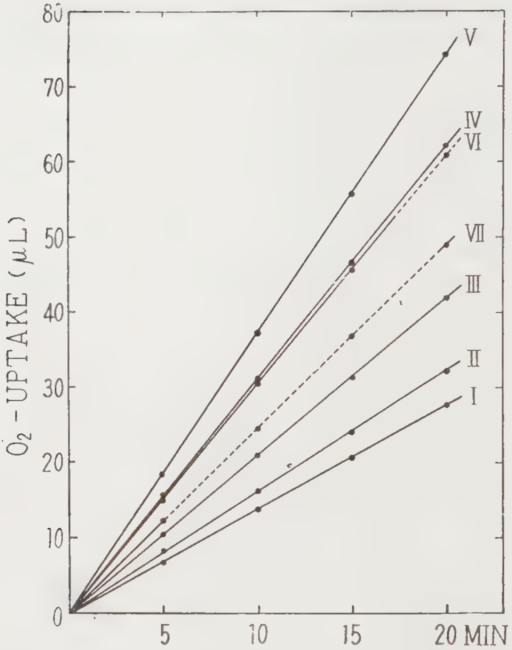

FIG. 8. Oxygen uptake in the presence of sodium benzoate.
Concentration of cytochrome c: 3.31×10^{-5} *M*.
Concentrations of Benzoate: I, 0.0 *M*; II, 0.25 *M*; III, 0.5 *M*; IV, 0.7 *M*; V, 1.0 *M*; VI, 1.5 *M*; VII, 1.75 *M*.
Concentration of ascorbic acid: 1.5×10^{-2} *M*.
pH: 7.4 (0.1 *M* phosphate buffer).

It was further investigated, why this promotion of oxidase activity is restricted in higher benzoate concentrations. For this purpose, the rate of the reduction of cytochrome c by ascorbate was investigated spectrophotometrically in the presence of benzoate in varied concentrations. Under the condition described in Fig. 10, the reduction rate was determined by measuring the optical desity at 550 mμ. As indicated in Fig. 10, benzoate in lower concentrations up to 1.0 *M* showed no appreciable effect upon the reduction rate. Above 1.0 *M*, however, the reduction rate was abruptly decreased. From these facts, it may be assumed, that the decrease of the promoting effect for the oxidase activity of the modified cytochrome c by benzoate above 1.0 *M* is to

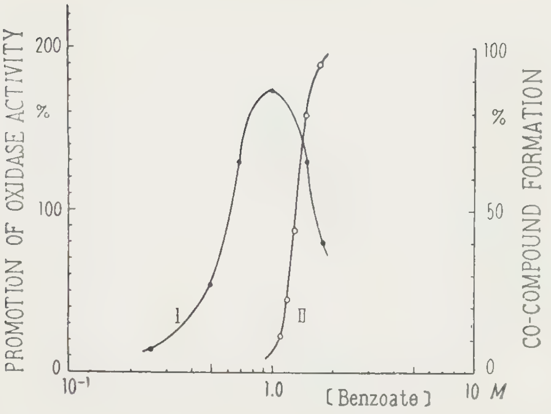

FIG. 9. Percentage representation of the promoting effect of benzoate upon the oidase activity.

I: Promotion of oxidase activity.

II: CO-compound formation.

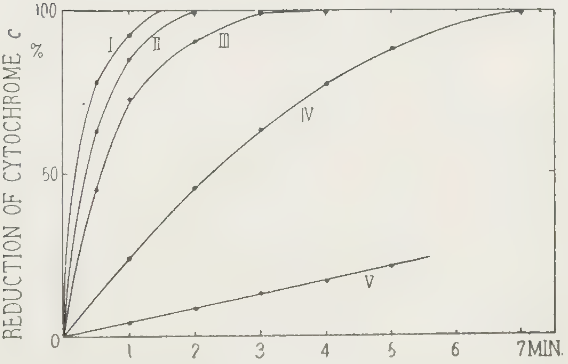

FIG. 10. Reduction of cytochrome c by ascorbic acid in the presence of varying benzoate concentrations.

Concentration of cytochrome c: 1.5×10^{-5} *M*.

Concentration of ascorbic acid: 1.7×10^{-2} *M*.

Concentrations of benzoate: I, 0.0–0.5 *M*; II, 1.0 *M*; III, 1.2 *M*;
IV, 1.3 *M*; V, 2.0 *M*.

pH: 7.0 (0. 1 *M* phosphate buffer).

T: 12.

be attributed to the decrease of the rate of the reduction of the modified cytochrome c by ascorbate.

The oxidase activity of the modified cytochrome c induced by 1.0 *M* benzoate was completely inhibited by addition of KCN. The rate of the reduction of the modified cytochrome c (with 1.0 *M* benzoate added) by ascorbate, when investigated spectrophotometrically, was found to be much slower in the presence of KCN than without it. This fact further supports the assumption that the decrease of the promotive effect on oxidase activity is due to the decreased reduction rate of modified cytochrome c by ascorbate.

Thus, it was evidenced that the protein modification in cytochrome c by benzoate addition proceeds stepwise. At the first step of the modification, the oxidase activity is significantly promoted prior to the second step, at which the formation of CO-compound is now possible. A similar series of experiments was undertaken using salicylate as the perturbator. As shown in Fig. 11, promotion of oxidase activity of cytochrome c preceded to the formation of CO-compound according to the increasing concentration of salicylate. The decrease of the oxidase promotion at higher salicylate concentration seems to be also duc to the restricted reduction rate of cytochrome c by ascorbate as already mentioned in the similar case with benzoate.

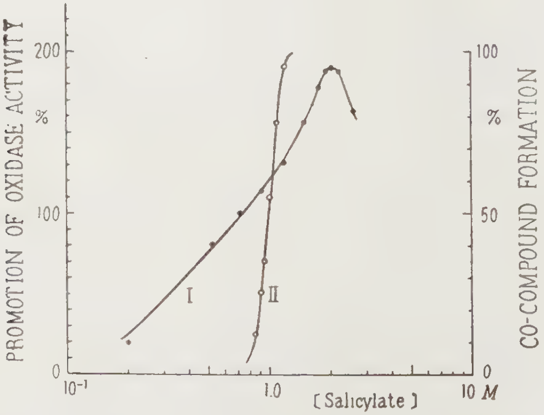

FIG. 11. Percentage representation of the promoting effect of sodium salicylate upon the oxidase activity.

I: Promotion of oxidase activity.

II: CO-compound formation.

DISCUSSION

In conclusion, we feel confident from the evidence presented here, that cytochrome c is modified in its protein portion through the effect of various perturbators, namely of salicylate, benzoate and lauryl sulfate and that the modification proceeds stepwise, where by the individual steps in the process can well be identified by their catalytic behaviour and their chemical characteristics. This step process of the modification was visualized in three stages at the different concentration ranges of perturbators. At the first stage of the modification of cytochrome c, a promotion of its oxidase activity appears. At the second stage, the formation of CO-compound is demonstrable and at the third and the final stage, the change occurs in its absorption spectra. Thus, according to the grade of the modification in its protein portion, the function of heme in the molecule of cytochrome c is altered stepwise.

The modification in the second stage, investigated by the formation of CO-compound as the criterion, is completely reversible, while that in the third stage is irreversible, at which the modification can be followed up by means of its absorption change. It is to be concluded thus, that the protein portion of this cytochrome c is affected ending to a irreversible modification through the reversible stages preceding. This sort of stepwise modification has already been presented in the experiment with hemoglobin and myoglobin in our laboratory (*1–4*). It is especially to be noted that the oxidase activity is definitely promoted at the perturbator concentration not yet efficient for the formation of CO-compound.

Pyridine hemochrome in solution, when saturated with CO-gas, formes CO-compound. It does not, however, at the high concentration of pyridine presented (*11*). This means that the bonding of pyridine and carbon monoxide to heme-Fe is competitive. Thus, when cytochrome c in reduced form is modified in its protein portion and becomes accessible to the bonding of carbon monoxide, the affinity of heme-Fe to the protein-N may be weakend as the result of protein modification. Pyridine hemochrome formed in the presence of pyridine in high concentration and not yet in the state capable of bonding with carbon monoxide, can even activate oxygen. Considering these facts, it may be assumed that there exists possibly a modification stage at which its oxidase activity is promoted preceding the stage delivering its CO-compound.

Kikuchi and Tomimura (*4*) have reported on their findings that the oxidase activity of hemoglobin was strongly promoted in the presence of benzoate or salicylate in very lower concentrations.

Considering all these findings, it is assumed that the essential function of heme in the individual hemeproteins may be subjected to a striking alteration even by a very slight grade of modification in their protein portion.

In respect to the absorption spectra, by saturation of carbon monoxide in the solution of reduced cytochrome c in the presence of benzoate, salicylate and lauryl sulfate, the spectra changed to the final figures as indicated in the Curves 4 V, 6 V, and 7 VI, respectively. These absorption figures may thus be assumed to be the final spectra of the compounds of heme-Fe and carbon monoxide. But these spectra are remarkably different with the ordinal spectra of the CO-compound of heme derivatives so far described in the literature.

With respect to the final absorption spectrum of the modified cytochrome c (Curve VI, Fig. 3) obtainable by the addition of lauryl sulfate in high concentration, Keilin (*12*) is in the assumption that this spectrum is to be accounted for the compound of protein and hydroxyethyl groups of heme moiety through thioether bonding after that the bonding of heme-Fe to protein-N has been dissociated off. But this spectrum also differs undoubtedly from that of free heme. It remains to be investigated further whether this deversity of the absorption figures is related with the difference in the side chains of heme.

SUMMARY

The modification of cytochrome c by the effect of benzoate, salicylate and lauryl sulfate was studied by measuring the spectral changes, CO-binding affinity and oxidatic activity as the criteria of the structural modification of its protein portion. The result obtained are as follows:

1. When benzoate was added to the solution of reduced cytochrome c, the latter became accessible to the bonding with carbon monoxide, which could be proved by its absorption spectra after the saturation of the solution with CO-gas. It was assumed thus, that sodium benzoate effects for the modification of the protein portion of cytochrome c. This stage of the modification was proved to be reversible.

The relation between the percentages of the formation of modified cytochrome c delivering CO-compound and the concentration of benzoate presented was indicated by a sigmoid curve of a high order reaction.

2. A similar result was obtained by using sodium salicylate in place of sodium benzoate. In this case, however, the modification of cyto-

chrome c occured in lower concentration than in the case with benzoate. At 0.8 *M* sodium salicylate, the absorption of the CO-compound of the modified cytochrome c reached the final figure.

Further increase of the salicylate concentration caused a diversed changes in the spectrum of reduced cytochrome c even without the presence of carbon monoxide. This absorption change could distinctly be observed also by the addition of lauryl sulfate. These absorption changes were proved to be irreversible. More serious modification seemed to be occuring, in this case, in the protein portion of cytochrome c.

3. Investigating the oxidase activity of modified cytochrome c with ascorbic acid as the substrate, the relation between the percentages of the promotion of oxidase activity and concentration of benzoate presented was obtained as a bell shaped curve. In the concentration range of added benzoate effective for the promotion of oxidase activity of thus modified cytochrome c, the formation of CO-compound of the latter in reduced form could not yet be proved. Above 1.0 *M* of benzoate, at which its CO-compound is now available, the percentage of the promotion of oxidase activity was decreased. This seems to be due to the decreased rate of the reduction of thus modified cytochrome c by ascorbate. A similar phenomenon could also be observed by salicylate, as already mentioned above.

The present authors wish to thank Prof. K. Kaziro for his encouragement and helpful discussions during the course of this work. This work was supported in part by the Scientific Research Fund of Ministry of Education for which we wish to express our thanks.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

TRANSAMINASES IN A HALOPHILIC BACTERIUM No. 101

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(Received for publication, July 30, 1956)

Few reports are available on the enzymochemical studies of the halotolerant and halophilic bacteria, although they represent a most interesting ecological group of organisms and are of some importance in food industries (*1*). On the other hand, the development of the studies on transaminases is very striking in recent several years, and their metabolic significance has been recognized considerably, but the problems of the ' multiplicyt ' of transaminases are open to discussion (*2*). The present authors wish to report some information involved in the transaminase activities of the cell-free extracts of a halophilic *Pseudomonas* strain No. 101 (*1, 3, 4*), especially in their ' multiplicity.'

METHODS

Preparation of Cell-Free Extracts—A halophilic *Pseudomonas* strain No. 101 was grown for 24–48 hours at 30° on a medium consisting of 1 per cent peptone broth and 10 per cent NaCl. The cells were harvested by centrifugation, washed twice with 10 per cent saline, and then lysed in distilled water. The cell débris was removed by centrifugation at $20,000 \times g$ for 30 minutes at low temperature.

Enzymatic Methods—To test enzyme preparations for transaminase activity, reaction mixtures were prepared in $0.1\ M$ phosphate buffer at pH 7.0 and they usually contained 0.25 ml of enzyme, 25 μM of α-ketoglutaric acid and 50 μM of L-amino acid in a final volume of 1 ml. The mixtures were incubated in stoppered tubes at 37° for the desired period of time usually 2 hours and the reactions were stopped by holding the tubes in boiling water for 5 minutes. L-Glutamic acid contained was then determined manometrically by decarboxylation with specific L-glutamic decarboxylase of *E. coli* Crookes strain (*5*).

RESULTS

Transaminase Activities of Crude Extracts—The data in Table I demonstrate some of the transamination reactions that occurred with α-keto-

TABLE I

*Transaminations with α-Ketoglutaric Acid Catalyzed by Crude Extracts**

Amino acid added	Relative value of L-glutamic acid formed	Amino acid added	Relative value of L-glutamic acid formed
None	7	Cystine "	–7
Aspartic acid	100	Methionine	6
Glycine	5	Lysine	–2
Alanine	7	Histidine	–2
Valine	9	Proline	2
Leucine	11	Hydroxyproline	5
Isoleucine	8	Phenylalanine	106
Serine	–5	Tyrosine "	18
Threonine	–6	Tryptophan "	3

* Reaction mixture contained 0.25 ml of enzyme, 25 μM of α-ketoglutaric acid, and 50 μM (or saturated solution) of L-amino acid as indicated in a final volume of 1.0 ml of 0.1 *M* phosphate buffer at pH 8.0. Incubated 2 hours.

glutaric acid when crude extracts of halophilic *Pseudomonas* strain No. 101 were used. The most active were L-aspartic acid and L-phenylalanine, and L-tyrosine showed also some activity in transaminating with α-ketoglutaric acid.

Effect of pH—The pH activity curves for crude extracts were also determined for the sake of comparing phenyalanine- and aspartic acid–α-ketoglutaric acid transaminase with each other and for comparison with the findings of other workers and show a pH maximum around 7.7 and 6.3 respectively. (Fig. 1)

Effect of NaCl Concentration—Plotting the relative activities against NaCl concentration, and taking the case in which no NaCl was added as 100, it was illustrated that, unlike alkaline phosphomonoesterase having a high optimum salt concentration of this halophilic bacterium (*3*), these enzymes were not halophilic. But it seemes to be more halotolerant than usualy. The typical experimental data are represented in Fig. 2.

Effect of Pyridoxal Phosphate—Dialysis for 20 hours against 0.01 *M* phosphate buffer at pH 7.0 resulted in considerable loss of the transaminase activities for aspartic acid, phenylalanine and tyrosine. This occurred also in the case of only storing the extracts in a refrigerator

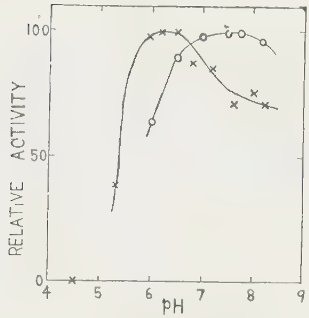

FIG. 1. Effect of pH on phenylalanine and aspartic acid transaminase activity.

Reaction mixture contained 25 μM of α-ketoglutaric acid, 50 μM of L-aspartic acid (—×—×—) or L-phenylalanine (—O—O—), and 0.25 ml. of enzyme in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH as given in the figure, Incubated 2 hours at 37°. Values obtained in absence of amino donor have been substracted to give above values.

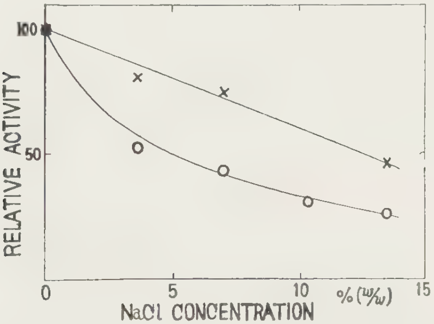

FIG. 2. Effect of NaCl concentration on phenylalanine and aspartic acid transaminase activity.

Reaction mixture contained 25 μM of α-ketoglutaric acid, 50 μM of L-aspartic acid (—×—×—) or L-phenylalanine (—O—O—) and 0.25 ml. of enzyme, and NaCl as given in the figure in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer pH 8.0. Incubated 2 hours at 37°.

for 20 hours. In the both cases, the addition of pyridoxal phosphate increased the activities considerably (Table II).

TABLE II

*Effect of Pyridoxal Phosphate on Transaminase Activities of Various Preparations**

Enzyme preparation	Pyridoxal phosphate	Amino acid-α-ketoglutaric acid transaminase activities (Q_{TN})		
		Aspartic acid	Phenylalanine	Tyrosine
Fresh extract (I)	−	805	1,490	385
	+	892	1,590	402
Prepn. I dialyzed 20 hours in refrigerator (0.01 *M* phosphate buffer, pH 7.0)	−	92	184	26
	+	590	850	236
Prepn. I stored 20 hours in refrigerator	−	61	88	−
	+	385	710	−

* Reaction mixture contained 0.2 ml. of enzyme, 30 μM of α-ketoglutaric acid, 50 μM of L-amino acid as indicated, and 50 μg. of crude pyridoxal phosphate (in the cases as indicated with +) in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH 7.0. Incubated 2 hours at 37°.

Effect of Heat-Treatment—Pretreatment of the extracts by heating at 55° for 10 minutes resulted in almost complete loss of the transaminase activities. Table III showes the effect of the heat-treatment at 48° on

TABLE III

Effect of Heat-Treatment of Enzyme Extracts at 48°*

Treatment time	Transaminase activities (Q_{TN})			Activity ratio	
	Aspartic acid (A)	Phenylalanine (P)	Tyrosine (T)	(A)/(P)	(T)/(P)
minutes					
0	792	1,360	211	0.58	0.16
10	274	950	211	0.29	0.22
30	116	243	42	0.48	0.17

* Reaction mixture contained 0.25 ml. of enzyme after treatment as indicated, 25 μM of α-ketoglutaric acid, and 50 μM of L-amino acid as indicated in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH 7.0. Incubated 2 hours at 37°.

the transminations between α-ketoglutaric acid and aspartic acid, phenylalanine and tyrosine. Such treatment reduced the activity of aspartic acid transaminase more effectively than that of phenylalanine- and tyrosine- transaminase at an early stage.

Transaminase Activities and Age of the Cultures—When cells are removed from a growing culture of the halophilic bacterium No. 101, extracted with distilled water, and tested for transaminase activities as described previously, aspartic acid-, phenylalanine- and tyrosine-transaminase activities and these ratios varied with the age of the culture as shown in Table IV a and IV b.

DISCUSSION

With comparison to the usual distributions of transaminase activities, it is of interest that in this case phenylalanine was as active as aspartic acid in transaminating with α-ketoglutaric acid, and alanine and all the others except for tyrosine showed little activities on the other hand. Moreover, it is suggested from Table III and IV that the transaminations of phenylalanine and aspartic acid with α-ketoglutaric acid are catalysed by the different enzymes respectively, because of the considerable differences of activity ratios. Thus in the cell-free extracts of this halophilic *Pseudomonas* strain No. 101, at least two L-amino acid–α-ketoglutaric acid transaminases exist predominantly, one corresponding

TABLE IV a

*Transaminase Activities and Age of the Culture**

Age of the culture	Transaminase octivities (Q_{TN})		Activity ratio (A)/(P)
	Aspartic acid (A)	Phenylalanine (P)	
hours 24	1,640	2,030	0.89
36	1,088	1,920	0.57
40	940	1,910	0.49
48	840	1,930	0.44

* Reaction mixture contained 0.25 ml. of enzyme extracts, 25 μM of α-ketoglutaric acid, and 50 μM of L-amino acid as indicated in a final volume of 1.0 ml. of 0.1 *M* phosphate buffer at pH 7.0. Incubated 2 hours at 37°.

TABLE IV b

*Transaminase Activities and Age of the Culture**

Age of the culture	Transaminase activities (Q_{TN})		Activity ratio (T)/(P)
	Phenylalanine (P)	Tyrosine (T)	
hours			
24	2.170	340	0.16
44	2,090	388	0.19

* Reaction mixture contained 0.25 ml. of enzyme extracts, 25 μM of α-ketoglutaric acid, and 50 μM of L-amino acid as indicated in a final volume of 1.0 ml. of 0.1 M phosphate buffer at pH 7.0. Incubated 2 hours at 37°.

to phenylalanine, the other to aspartic acid.

SUMMARY

1. Water-lysed extracts of a halophilic *Pseudomonas* strain No. 101 catalyzed the transamination reactions between α-ketoglutaric acid and L-aspartic acid, L-phenylalanine and L-tyrosine (maximum Q_{TN}: 1,640, 2,170 and 388, respectively).

2. These transaminases were not halophilic unlike alkaline phosphomonoesterase of this halophilic bacterium.

3. From the activity changes during heat-treatment and growth of the culture, it was suggested that at least two transaminases existed in the lysed extracts, one corresponding to aspartic acid and the other to phenylalanine.

The authors are indebted to Prof. F. Egami, Chemical Institute, Faculty of Science, Nagoya University, for the gift of the bacterial strain and also to Mr. M. Shiroishi, Food Research Institute, the Department of Agriculture and Forestry, for the preparation of pyridoxal phosphate.

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ÜBER PFLANZENPROTEASEN

VI.[1] DIE PROTEOLYTISCHE AKTIVITÄT DES SOJA-LEGUMELINS

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(Der Schriftleitung zugegangen am 1 August, 1956)

Shon vor mehreren Jahren haben wir (*2*) unter Anwendung der Wasserextraktionsmethode die Darstellung einer Protease aus den Samen von Sojabohne (*Glycine Max Merrill*) versucht, aber nur mit negativem Erfolge, indem das Vorkommen dieses Enzyms in Keimpflanzen bestätigt wurde. In den letzten Jahren wurden inzwischen einige Protease-Inhibitoren von mehreren Forschern, wie z. B. das Chymo-Inhibitor von H. Tauber (*3*), das Trypsin-Inhibitor von M. Kunitz (*4*) und das Antipapain-Faktor von E. M. Learmonth (*5*), im Wasserauszuge von Sojabohnen aufgefunden. In Berücksichtigung dieser Umstände haben wir nun die frühere Aufgabe wieder uns aufgestellt, um damit auf die Frage zu beantworten, ob ein wirksames Enzym schon in ungekeimten Samen fertig enthalten sei oder nicht.

Zur Entfernung der Inhibitoren haben wir ein zerriebenes Samenpulver mit Wasser ausgezogen, und das dabei zurückgebliebene Kuchen in Toluolwasser zur Autolyse unterworfen. Hierbei liess sich das Leucin in guter Ausbeute aus dem Digestat gewinnen, während der Kontrollversuch mit dem zuvor nicht abgeschlämmten Samenpulver immer ein negatives Resultat gab. Daher liegt es nahe anzunehmen, daß eine sonst glatt vor sich gehende Proteolyse in Samen durch einem beigemischten extrahierbaren Inhibitor einer starken Herabsetzung erleiden mag. Bei dieser Sachlage haben wir dann die Veränderung des scheinbaren Proteasengehaltes in Samen bei der Keimung verfolgt. Um einen Entwicklungsgrad der Pflanze in sicherer Weise auszudrücken, haben wir einerseits die Länge der Keimlinge gemessen, und andererseits das Gesichtsverhältnis des Axenorgans zum gesamten Pflänzchen bestimmt. Zur Darstellung der Enzymlösung wurden die Pflanzen in Kotyledonen und Axenorganen abgeteilt, und bei den ersteren wurde das zerriebene

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* z. Zt.: Das Botanische Laboratorium, Fakultät der Wissenschaft der Universität zu Tokyo.

Pflanzenmaterial unter Zusatz von gleicher Menge des Toluolwassers autolysiert, und die filtrierte Flüssigkeit wurde als Enzymlösung angewandt. Bei den Axenorganen wurde die ausgepresste Lösung, nach der Filtration, zur enzymatischen Prüfung herangezogen. Zur Ermittelung der proteolytischen Aktivität haben wir die Gelatinespaltung durch Titrationsmethode nach R. Willstätter (*6*) bestimmt. Die Versuche mit den aus Kotyledonen dargestellten Enzymlösungen ergaben, daß die proteolytische Aktivität der Samen im Verlaufe des Keimungsprozesses vom Null über ein Maximum bis zum Minimum verändert wird. Und zwar der Maximalbetrag wurde an den Keimpflanzen von 6-täglicher Vegetationsdauer, deren Länge zu etwa 20 cm. und deren Gewichtsverhältnis zu 50–60 Proz. entspricht, angetroffen. Die Versuche mit den aus Axenorganen gewonnenen Lösungen zeigten, daß eine sehr träge Aktivität erst in späteren Keimungsphasen auftritt. Ausserdem konnten wir auf dem Autolysat der Kotyledonen von 6-tgl. Keimpflanzen das Leucin, und aus dem Extrakt der Axenorganen derselben Keimungsphase das Asparagin nebst Leucin gewinnen.

Durch die oben angeführten Tatbestände wurde es festgestellt, daß ein proteolytisches Enzym sowohl in Keimpflanzen als auch in ruhenden Samen enthalten ist. Hierbei bleibt aber die Frage noch offen, was für Gründe darin liegen, daß das Enzym in Samen unwirksam verschlossen ist und erst nach dem Auslaugen mit Wasser in aktive Form umgewandelt wird. In Anbetracht dessen haben wir nun die Fraktionierung der Proteine aus Samen oder Keimpflanzen unternommen. Als Ausgangsmaterialien dienten der Rückstand, der aus ruhenden Samen durch Abschlämmen mit Wasser vorbereitet ist, und das Autolysat, welches aus Kotyledonen der Keimpflanzen gewonnen ist, und sogar das aus unreifen Samen dargestellte Pulver. Aus diesen Substanzen haben wir das Glycinin und Legumelin nach dem Verfahren von T. B. Osborne und seinen Mitarbeitern (*7*) dargestellt. Unter Berücksichtigung der Umstände, daß sich das Legumelin, wie von diesen Autoren hingewiesen, bei der Berührung mit Alkohol oder Aceton leicht ins Unlösliche umwandeln lässt, haben wir das Albumin aus dessen wässrigen Lösung unter Toluolzusatz im Exsikkator über Schwefelsäure getrocknet. Daneben haben wir das Trypsininhibitor aus dem Wasserauszug der ungekeimten Samen nach dem modifizierten Verfahren von Kunitz' scher Methode dargestellt. Die proteolytische Wirksamkeit dieser Proteinpräparate wurde mit Gelatine geprüft. Dabei hat es sich ergeben, daß das Legumelin enzymatisch aktiv ist, während das Glycinin und Trypsininhibitor ganz unwirksam sind. Die inhibitorische Wirkung des Trypsininhibitors wurde beim Trypsinversuche mit Caseinspaltung und beim Papainversuche mit Gelatinespaltung verfolgt, mit dem Ergebnis, daß das Trypsininhibitor nicht nur gegen das Trypsin, sondern auch gegen das Papain einen spezifischen Hemmungskörper darstellt.

Um die enzymatische Natur des Soja-Legumelins zu erkennen, haben wir seine proteolytischen Wirkungen gegen Gelatine, mit besonderer Berücksicht auf ihre pH-Abhängigkeit und auf ihre Beeinfussbarkeit durch Blausäure oder Trypsininhibitor, näher studiert. Die Versuche ergaben, daß das Legumelin jeder Herkunft fast in gleichem Masse die Gelatine in optimalem pH-Bereiche 5.0–6.0 anzugreifen imstande ist, und daß diese enzymatische Aktivität des Legumelins durch Cyanhydrin gar nicht beeinflußt wird, aber durch Trypsin- oder Papaininhibitor stark gehemmt wird. Daraus geht es klar hervor, daß dies Protein nichts anderes als eine Proteinase von Papain- oder Kathepsintypus darstellt. Zur Charakterisierung der Papainasen im allgemeinen ist es sehr wichtig, wie schon von uns (*8*) dargelegt, die Erfahrungen über den Einfluß der Denaturierung der Proteine auf ihre enzymatische Spaltbarkeit in richtiger Erwägung zu ziehen. Hiermit haben wir nun die Spaltungsversuche mit den beiden Soja-Proteinen, Glycinin und Trypsininhibitor ausgeführt. Dabei wurde es nachgewiesen, daß das Glycinin, ungekochtes oder gekochtes, ein gutes Substrat für das Legumelin darstellt, während es beim Zusatz des Trypsininhibitors fast gar nicht angegriffen wird. Beim Versuch mit Trypsininhibitor ergab es sich, daß sich das native Protein gegen den Angriff des Legumelins ganz refraktär verhält, aber das Hitze-denaturierte durch das Enzym leicht aufgespalten wird. Dasselbe traf auch am Legumelin. Dies Enzymprotein zeigte in nativem Zustand keine Autolysierbarkeit, während es nach der Denaturierung durch Erhitzen leicht ins gute Substrat für das eigne Enzym umgewandelt wurde. Um weiter eine peptidatische Wirkung des Legumelins kennenzulernen, haben wir Asparagin, Benzoylglycin, Benzoylglycylglycin, Leucylglycin und Leucylglycylglycin zum enzymatischen Versuch unterworfen, wobei sich keine Spaltung ergab.

EXPERIMENTELLES

I. Beweise für die Existenz der Protease in den Samen und Keimpflanzen von Sojabohne

(1) Abhängikgket der proteolytischen Aktivität der Keimpflanzen von

ihren Entwicklungsphasen—Bestimmung der Entwicklungsgrade: Die Pflanzen wurden in großen, mit feuchtem Sand gefüllten Kästen unter Lichtabschluß bei Temp. 25–32° in Gewächshaus gezogen. Nach bestimmter Vegetationsdauer wurden 20 Keimlinge je in einem Versuch geerntet, und ihre mittelmässige Länge wurde gemeßen. Um den Translokationsgrad der Reservestoffe zu ermitteln, wurde das Gewichtsverhältnis eines Axenorgans zum gesamten Pflänzchen bestimmt. Darstellung der Enzymlösung: Die Kotyledonen der Keimpflanzen wurden von Axenorganen abgetrennt und zerrieben, die Masse wurde mit gleicher Menge des Wassers versetzt und unter Toluolschicht bei Zimmertemperatur 3 Tage aufbewahrt. Darnach wurde das Digestat filtriert, und das klare Filtrat wurde als Enzymlösung verwendet. Bei den Axenorganen wurden die zerquetschte filtrierte Flüssigkeit bediente zu diesem Zweck. Bestimmung der proteolytischen Aktivität: In 1 ml. Ansatz sind 60 mg. Gelatine, 0.4 ml. Enzymlösung (pH 6.0) und einige Tropfen Toluol enthalten. Temp. 30°. Versuchsdauer 24 Stdn. Die COOH-Zunahme wurde durch Titrationsmethode nach Willstätter mittelst 0.025 *N* KOH bestimmt. Die Ergebnisse sind in Fig. 1 veranschaulicht.

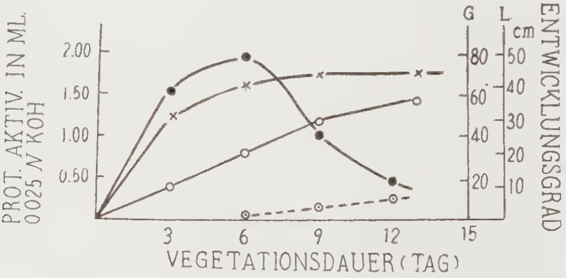

FIG. 1. Abhängigkeit der proteolytischen Aktivität der Keimpflanzen von ihrem Entwicklungsgrad.

L. —○—○—, Länge des Axenorgans; G. —×—×—, Gewichtsverhältnis; —●—●—, Proteolytische Aktivität der Kotyledonen; —◉—◉—, Proteolytische Aktivität der Axenorgane.

(2) *Isolierung der Aminosäuren aus den Keimpflanzen und aus dem Autolysat der ungekeimten Samen*—Asparagin und Leucin aus Axenorganen: 5982 g. Axenorgane der 6-tgl. Keimpflanzen wurden ausgepresst, der Rückstand wurde abermals mit 1 L. heissem Wasser ausgezogen. Der ver-

einigte Extrakt wurde auf Wasserbad aufgekocht, und die vom dabei abgeschiedenen Koagulum befreite Flüssigkeit wurde unter vermindertem Druck bis zu etwa 200 ml. eingeengt und in der Kälte überlassen, wobei sich die Asparaginkrystalle in reichlicher Menge abschieden. Aus der eingeengten Mutterlauge wurde eine weitere Krystallization dieses Amides ermöglicht. Das Rohmaterial wurde zweimal aus heissem Wasser umkrystallisiert, dann die reine Substanz wurde in einer Ausbeute von 55 g. erhalten. Die Mutterlauge des Asparagins wurde im Vakuum verdampft, der syrupöse Rückstand wurde mit heissem Methylalkohol ausgezogen, und der Extrakt wurde mit Äther versetzt. Der dabei abgeschiedene Fällungskörper wurde aus 70 proz. Alkohol umkrystallisiert, und das Leucin wurde als feine Krystalle dargestellt. Ausbeute 4.5 g. Asparagin und Leucin aus Kotyledonen der Keimpflanzen: 1195 g. Kotyledonen der 6-tgl. Keimlinge wurden im Mörser zerrieben und mit 1 L. heissem Wasser ausgezogen. Aus dem eingeengten Filtrat wurde das Asparagin in der Menge von 2.5 g. gewonnen. Die Mutterlauge wurde im Vakuum verdampft, der Rückstand wurde mit Methylalkohol ausgezogen, der Extrakt wurde mit Äther versetzt, und das dabei abfallende Leucin wurde aus 70 proz. Alkohol umkrystallisiert. Ausbeute des reinen Präparates betrug 2 g. Leucin aus dem Autolysat der ruhenden Samen: 500 g. Samen wurden mit Wasser aufgequollen, enthüllt und zerrieben, die Masse wurde mit 2 L. Wasser zugesetzt und kräftig durchgeschüttelt, und die Aufschwemmung wurde auf Seihtuch ausgepresst. Der Rückstand wurde in 2 L. Wasser verteilt und unter Toluolzusatz bei 30° eine Woche überlaßen. Das Autolysat wurde filtriert, und der Rückstand wurde abermals mit 1 L. Wasser ausgezogen. Der vereinigte Extrakt wurde auf Wasserbad aufgekocht. Die vom dabei entstandenen Koagulum abfiltrierte Flüssigkeit wurde unter vermindertem Druck bis zu etwa 100 ml. eingeengt und darauf mit gleichem Volumen des Alkohols versetzt, wobei sich eine peptonartige Substanz abfallen ließ. Das alkoholische Filtrat wurde abgedampft, der zurückgebliebene Syrup wurde mit Methylalkohol ausgezogen. Der methylalkoholische Extrakt wurde mit Äther zugesetzt, dann das Leucin schied sich in pulverigem Zustand ab. Das Rohmaterial wurde aus 70 proz. Alkohol umkrystallisiert, und das reine Leucin wurde in der Ausbeute von 1.2 g. erhalten.

An den oben erwähnten Substanzen wurde der Gesamt-N nach mikro-Kjeldahl, der NH_2-N nach van Slyke und der COOH-N nach Willstätter bestimmt. Die Ergebnisse sind in Tab. I zusammengestellt.

TABELLE I

Analytische Daten des Asparagins und des Leucins

Asparagin	H_2O	Gesamt-N	NH_2-N	COOH-N
	%	%	%	%
Ber. für $C_4H_8N_2O_3 \cdot H_2O$	12.00	18.67	9.34	9.34
Gef. am Präp. aus Axenorganen	12.08	18.72	9.38	9.52
Leucin		**Gesamt-N**	**NH_2-N**	**COOH-N**
		%	%	%
Ber. für $C_6H_{13}NO_3$		10.69	10.69	10.69
Gef. am Präp. aus Kotyledonen der Keimpflanzen		10.53	10.66	10.50
„ „ „ „ Axenorganen „ „		10.62	10.85	10.50
„ „ „ „ Autolysat der samen		10.52	10.69	10.74

II. Darstellung der Proteine aus Samen und Keimpflanzen

(*1*) *Glycinin* — Ruhende Samen: Der Pressrückstand, der aus 500 g. Samen nach dem Auslaugen mit Wasser gewonnen war, wurde mit 2 L. 10 % NaCl-Löung zweimal extrahiert. Die filtrierte Lösung wurde unter Toluolzusatz im Cellophanmembran gegen Leitungswasser dialysiert. Der dabei abgeschiedene Niederschlag wurde abermals in 5 % NaCl-Lösung gelöst und dialysiert. Das umgefällte Glycinin wurde auf Filter gesammelt, mit Wasser nachgewaschen und durch Behandlung mit Alkohol und Äther getrocknet. Ausbeute betrug 40 g. Kotyledonen der Keimpflanzen: 1825 g. Kotyledonen der 6-tgl. Keimpflanzen (entsprechend 840 g. Samen) wurden zerrieben, in 2 L. Wasser verteilt und unter Toluolzusatz bei Zimmertemperatur 5 Tage aufbewahrt. Das Digestat wurde auf Wollentuch und dann auf Filtrierpapier filtriert. Die klare Flüssigkeit wurde mit Ammoniumsulfat gesättigt. Der dabei ausgesalzene Eiweisskörper wurde in Wasser aufgelöst und abermals dialysiert. Das abgeschiedene Globulin wurde durch Behandlung mit Alkohol und Äther getrocknet. Ausbeute 11 g. Unreife Samen: 2800 g. der enthüllten grünen Samen wurden in Reibschale fein gestossen, und mit 9 L. 10% NaCl-Lösung ausgezogen. Der filtrierte Extrakt wurde mit Ammoniumsulfat gesättigt. Der abgeschiedene Niederschlag wurde in Wasser gelöst und dialysiert, wobei sich das Globulin in einer Ausbeute von 35 g. abfallen.

(*2*) *Legumelin oder Proteinase* — Präp.–I aus ungekeimten Samen: 6 L. der dialysierten Lösungen, welche bei der Darstellung des Glycinins aus 500 g. Samen erhalten waren, wurden mit Ammoniumsulfat gesättigt. Der Fällungskörper wurde in 500 ml. Wasser aufgelöst und dialysiert. Das filtrierte Dialysat wurde mit Natriumsulfat gesättigt, und die Aussalzung wurde in Wasser umgelöst und dialysiert. Die Flüssigkeit wurde nach der Filtration unter Toluolzusatz im Exsikkator über Schwefelsäure getrocknet, wobei sich das Legumelin in genuinem Zustand darstellen liess. Ausbeute betrug etwa 2 g. Präp.–II aus Kotyledonen der Keimpflanzen: 1427 g. Kotyledonen der 6-tgl. Keimlinge (entsprechend 656 g. Samen) wurden pulverisiert, mit 1 L. 10% Na_2SO_4-Lösung versetzt und unter Toluolschicht bei Zimmertemperatur 3 Tage stehen gelassen. Das Digestat wurde auf Seihtuch ausgepreßt, und der Rückstand wurde abermals mit 1 L. 10% Na_2SO_4-Lösung behandelt. Der vereinigte Auszug wurde mit Natriumsulfat gesättigt. Die Abscheidung wurde mit 200 ml. Wasser aufgenommen, und die vom ungelösten Körper befreite Flüssigkeit wurde mit 50 g. Natriumsulfat versetzt. Der dabei erhaltene Niederschlag wurde in Wasser gelöst und dialysiert. Das filtrierte Dialysat wurde im Vakuum getrocknet, dann das Legumelin wurde in nativem Zustand gewonnen. Ausbeute 1.5 g. Präp.–III aus unreifen Samen: 2800 g. Kotyledonen wurden zerrieben und mit 9 L. 10% NaCl-Lösung ausgezogen. Dieser Extrakt wurde durch Dialyse vom Glycinin befreit, und das Filtrat wurde mit Natriumsulfat gesättigt. Der Fällungskörper wurde in Wasser gelöst und dialysiert, das Dialysat wurde wie üblich getrocknet, und das Legumelin wurde in der Ausbeute von 2 g. erhalten.

(3) *Trypsininhibitor* — 850 g. Samen wurden mit Wasser aufgequollen, enthüllt und zerrieben. Die Masse wurde mit 3 L. Wasser zugesetzt und durch einen Mull unter Handpresse ausgetrieben. Die Presssaft wurde filtriert, und die Lösung wurde mit Ammoniumsulfat gesättigt. Der Fällungskörper wurde mit 200 ml. Wasser aufgenommen, und die von unlöslicher Substanz befreite Flüssigkeit wurde dialysiert. Das filtrierte Dialysat wurde mit 0.25 N H_2SO_4 zugesetzt, um damit die Azidität der Lösung auf pH 5.4–5.6 eingestellt zu werden. Die dabei entstandene Abscheidung wurde entfernt, und die filtrierte Flüssigkeit wurde unter weiterem Zusatz von 0.25 N H_2SO_4 auf pH 4.4 gebracht. Die abgeschiedene Fällung wurde in wenig Wasser verteilt, und durch Zusatz von 0.25 N NH_4OH auf pH 5.8 eingestellt, wobei eine fast vollständige Auflösung erfolgte. Der rohe Inhibitor wurde durch mehrmaliges Abfällen bei pH 4.4 und nachheriges Auflösen bei pH 5.8 gereinigt. Die zuletzt dargestellte Proteinlösung wurde mit Aceton zugesetzt, und

der Fällungskörper wurde auf Glasfilter gesammelt, mit Alkohol und Äther behandelt, und im Exsikkator über Schwefelsäure getrocknet. Ausbeute 0.65 g. Die inhibitorische Wirksamkeit dieses Präparates wurde in folgender Weise bestimmt: Beim Trypsin-Versuch sind in 1 ml. Ansatz 50 mg. Casein, 2 mg. Trypsin-Proteinase und 0.4 mM. NH_4OH (pH 8.8) enthalten, und beim Papain-Versuch sind in 1 ml. Ansatz 60 mg. Gelatine und 5 mg. Papain (pH 6.0) vorhanden. In allen Versuchen wurde Toluol als Antiseptikum zugesetzt. Temp. 30°. Zur Bestimmung der Aktivität wurde die COOH-Zunahme durch Titrationsmethode mittelst 0.025 *N* KOH gemessen. Die Ergebnisse sind in Tab. II veranschaulicht.

TABELLE II

Inhibitorische Aktivität des Trypsininhibitors gegen Trypsin- bzw. Papainwirkung

Zusatz von Trypsininhibitor	Trypsin (2 mg.) Versuchsdauer (Stdn.)			Papain (5 mg.) Versuchsdauer (Stdn.)		
	1	4	24	3	6	24
mg.	ml.	ml.	ml.	ml.	ml.	ml.
0.0	1.90	4.00	5.20	0.90	1.10	1.80
2.0	0.20	1.00	2.20	0.30	0.50	0.70

Zur Charakterisierung der oben angeführten Soja-Proteine wurde der Gesamt-N nach mikro-Kjeldahl, der NH_2-N nach van Slyke, und der COOH-N nach Willstätter bestimmt. In folgender Tabelle sind die Ergebnisse zusammengestellt.

III. Proteolytische Aktivität des Legumelins

(*1*) *Proteinasewirkung*—Substratproteine: Gelatine und Soja- Proteine sind angewandt, und die Soja-Proteine wurden unter Umständen durch Aufkochung mit Wasser während 30 Minuten denaturiert. Anstellung der Versuche: Beim Gelatine-Versuch sind in 1 ml. Ansatz 60 mg. Substratprotein, wechselnde Menge des HCl-NH_4OH-Puffers und 10 mg. Enzymprotein enthalten, und beim Versuch mit Soja-Proteinen sind in 1 ml. Ansatz 50 mg. Substratprotein und 10 mg. Enzym hineingemischt. Toluol als Antiseptikum. Temp. 30°. Versuchsdauer: 24, 72 Stdn. Die pH-Werte wurden kolorimetrisch bestimmt. Bestimmung der Aktivität: Die COOH-Zunahme wurde

Tabelle III

Analytische Daten der Soja-Proteine

Protein (lufttrocken)	Gesamt-N	NH_2-N	COOH-N
	%	%	%
Glycinin:			
Präp. aus ungekeimten Samen	13.50	0.23	0.41
Präp. aus Kotyledonen der Keimpflanzen	13.89	0.28	0.63
Präp. aus unreifen Samen	13.46	0.23	0.41
Legumelin (od. Proteinase):			
Präp.–I	13.09	0.56	1.75
Präp.–II	13.09	1.01	1.68
Präp.–III	13.20	0.79	1.82
Trypsininhibitor	13.33	0.56	0.84

nach Willstätter mittelst 0.025 *N* KOH gemessen. Die Ergebnisse sind in Tab. IV und Fig. 2 angegeben.

(*2*) *Peptidasewirkung*—Substrate: Das Asparagin wurde sus Soja-Keimpflanzen dargestellt, und die Polypeptide wurden nach der Vorschrift von E. Fischer (*9*) synthesiert. Anstellung der Versuche; In 1 ml. Ansatz sind 0.05 mM. Substrat, verschiedene Menge des Ammoniaks als Puffer und 10 mg. Enzym enthalten. Temp. 30°. Versuchsdauer 24 Stdn. Bestimmung der Aktivität: Wie oben. Die Ergebnisse sind in Tab. V zusammengestellt.

ZUSAMMENFASSUNG

Das Legumelin wurde aus ungekeimten Samen, Kotyledonen der Keimpflanzen und unreifen grünen Samen von Sojabohne in nativem Zustand dargestellt, und seine proteolytischen Wirkungen wurden mit den verschiedenen Substraten wie Gelatine, Glycinin, Asparagin, Hippursäure, Benzoylglycylglycin, Leucylglycin und Leucylglycylglycin im variierenden pH-Bereiche geprüft. Dabei hat es sich ergeben, daß das Legumelin der Kategorie von Papain- oder Kathepsin-Proteinase angehört, und völlig frei von jedweder peptidatischen Wirkung dargestellt ist. Ausserdem wurde es festgestellt, daß die Proteinase durch den Zusatz des Trypsininhibitors ihre Aktivität verliert, und daß dies Enzymprotein in aktivem Zustand nie einer Autolyse unterliegt, aber nach der Denaturierung leicht ins geeignete Substrat für dasselbe Enzym

TABELLE IV

Spaltbarkeit der Proteine durch Glycine-Proteinase

Substratprotein	Zusatz als Puffer, Aktivator oder Inhibitor (pro 1 ml. Ansatz)	pH	COOH-Zunahme in ml. 0.025 *N* KOH (nach 24 Stdn.) durch		
			Präp.-I	Präp.-II	Präp.-III
	mM.		*ml.*	*ml.*	*ml.*
Gelatine	HCl, 0.04	3.2–3.4	0.50	0.80	0.70
”	—	5.8–6.0	1.50	1.50	1.50
”	NH_4OH, 0.02	7.2–6.8	1.20	1.20	1.30
”	” , 0.05	8.2–7.6	—	0.80	1.00
”	” , 0.10	9.2–8.6	—	0.55	0.80
”	HCN, 0.01	6.0	1.20	1.40	1.40
”	Trypsininhibitor, 5 mg.	6.0	0.50	0.40	0.50
Glycinin, ungekochtes	—	6.0	0.90	0.90	1.00
” , gekochtes	—	6.0	—	0.90	—
” , ungekochtes	Trypsininhibitor, 5 mg.	6.0	0.10	—	0.20
Trypsininhibitor, ungekochtes	—	6.0	—	0.00	0.00
” , gekochtes	—	6.0	—	0.40	0.40
Enzymprotein, natives	—	6.0	0.00	0.00	0.00
” , denaturiertes	—	6.0	1.00	1.00	1.30

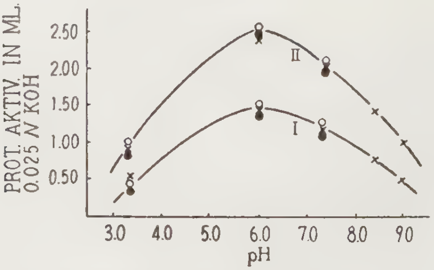

FIG. 2. pH-Abhängigkeit der Gelatinespaltung durch *Glycine*-Proteinase.

I, nach 24 Stdn.; II, nach 72 Stdn.; —●—●—, Präp.–I; —×—×—, Präp.–II; —○—○—, Präp.–III.

TABELLE V

Spaltbarkeit der Polypeptide durch Glycine-Proteinase

Substrat	Zusatz des Ammoniaks	pH	COOH-Zunahme in ml. 0.025 *N* KOH (nach 24 Stdn.) durch		
			Präp.-I	Präp.-II	Präp.-III
	*m*M		*ml.*	*ml.*	*ml.*
Asparagin	0.04	7.8	0.00	0.00	0.00
Benzoylglycin	0.20	5.0	0.00	0.00	0.00
Benzoylglycylglycin	0.20	5.0	0.00	0.00	0.00
Leucylglycin	0.06	8.2	0.00	0.00	0.00
Leucylglycylglycin	0.04	7.4	0.00	0.00	0.00

verändert werden kann.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

STUDIES ON THE OXIDATIVE DECOMPOSITION OF UROCANIC ACID

III. ISOLATION OF SUCCINIC MONOUREIDE*

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(Received for publication, August 2, 1956)

In previous papers (*1, 2*) oxidative decomposition of urocanic acid was found by the experiment carried out in the presence of EDTA or using the enzyme materials preserved over two weeks. This pathway differs from that of urocanic acid decomposition to glutamic acid. As the latter is preferential, the oxidative pathway is apt to be overlooked, when enzyme solutions are prepared from fresh tissues or cells. In the investigations on the oxidative product of urocanic acid, the authors have isolated succinic monoureide.

EXPERIMENTAL

Enzyme Solution—Pseudomonas aeruginosa was cultivated at 37° in neutral broth media in which histidine monohydrochloride was added in a concentration of 0.04 per cent. After 18 hours the cells were harvested with Sharples centrifuge and washed with *M*/50 phosphate buffer (pH 7.0) until the washing no longer gave the Pauly's diazo-reaction. The cell brei was dried with acetone. 40 g. of cell powder was ground with an equal amount of the sea sand, extracted with 1200 ml. of *M*/20 phosphate buffer (pH 8.0) for one hour in a refrigerator, and centrifuged at 3500 × *g* at 5° for 15 minutes. The supernatant was used as the enzyme solution.

EDTA—1.2 g. of EDTA was dissolved in 100 ml. of distilled water and the solution was adjusted to pH 8.0 with saturated sodium hydroxide (10^{-3} *M* EDTA in final concentration).

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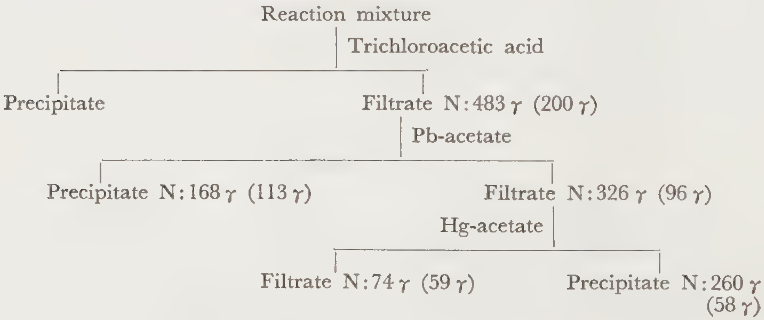
* This work was supported by a grant for scientific research from the Ministry of Education. We should like to express our hearty gratitude.

Enzymatic Decomposition of Urocanic Acid—The EDTA and the enzyme solutions were combined, 1 *M* phosphate buffer 150 ml. (pH 8.0) was added and the total amount was made up to 3000 ml. with distilled water, and to 2500 ml. of this mixed solution was added 5 gm. of urocanic acid (dissolved in 500 ml. of distilled water and adjusted to pH 8.0 with saturated sodium hydroxide). Equal portions (150 ml.) were placed in 500 ml. flasks, 1 ml. toluol was added to each and the flasks were shaken in an incubator at 37° for 13 hours. As controls, 500 ml. of the mixture of enzyme and EDTA solutions and 100 ml. of distilled water (without addition of urocanic acid) were used.

Isolation of Decomposition Product—Ater confirmation of the disappearance of the absorption spectrum of urocanic acid (268 mμ), the reaction was stopped by addition of trichloroacetic acid to the final concentration of 5 per cent. The deproteinized solution was treated as follows, and the nitrogen content of each fraction was traced (Scheme 1).

Scheme 1

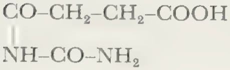

Reaction mixture
Trichloroacetic acid
Precipitate
Filtrate N:483 γ (200 γ)
Pb-acetate
Precipitate N:168 γ (113 γ)
Filtrate N:326 γ (96 γ)
Hg-acetate
Filtrate N:74 γ (59 γ)
Precipitate N:260 γ (58 γ)

This nitrogen values were corrected for 1 ml. of deproteinized solution.
( ) . . . Control-experiment.

Solid lead acetate was added to the solution until no more precipitate appeared. The filtrate was adjusted to pH 5.8 with sodium hydroxide and 20 per cent mercuric acetate (dissolved in 2 per cent solution of acetic acid) was added, pH being maintained by sodium hydroxide addition, until no more yellow precipitate appeared. The mixture was kept in the refrigerator overnight. The precipitate was gathered by suction and suspended in distilled water and decomposed with hydrogen sulfide. The filtrate, removed from mercury, was evaporated in vacuum at 60°. When the solution was concentrated to 20–30 ml. and cooled, 0.3 g. of yellowish white scaly crystal was obtained. It was recrystallized from warm water and decolorized with charcoal.

The white scaly crystal is sparingly soluble in cold water and alcohol, and soluble in warm water. The m. p. was 214° and this coincided with that of the known sample and a mixture of the two. It gave the carbamide reaction of Fearon (*3*). Analytical data are as follows:

		C %	H %	N %
	Found	37.95	5.08	16.45
		37.50	5.69	16.78
		37.79	5.04	17.11
$C_5H_8N_2O_4$	Theoretical	37.50	5.04	17.50


$$\begin{array}{l} CO\text{–}CH_2\text{–}CH_2\text{–}COOH \\ | \\ NH\text{–}CO\text{–}NH_2 \end{array}$$

The infra-red spectra of the isolated and the synthetic were essentialy the same (Fig. 1).

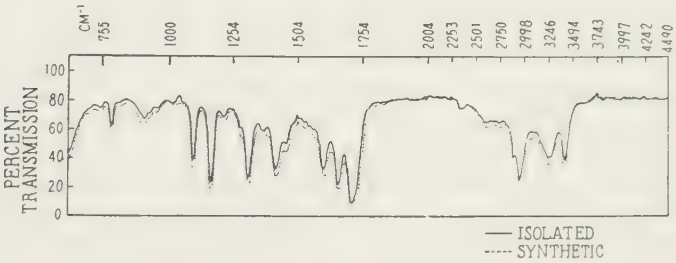


FIG. 1. Infra-red absorption spectra were made with Nujol-mull with a Hilger H 800 I. R. Spectrophotometer.
1: isolated, 2: synthetic succinic monoureide.

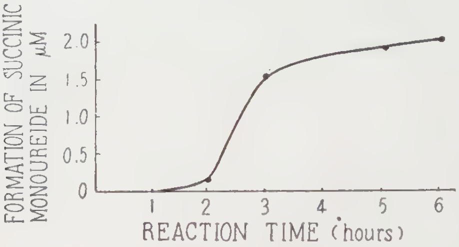
Succinic monoureide was not formed in the absence of urocanic acid (Control-experiment).

Succinic Monoureide Formation and Reaction Time—As shown in Fig. 2, 2 μM of succinic monoureide was formed within 5–6 hours from 30 μM of urocanic acid by the extract of Pseudomonas aeruginosa, when EDTA was present, and there was no further increase.

With the boiled enzyme solution and EDTA, any decrease of urocanic acid, oxygen-uptake, or production of succinic monoureide was not found. According to Stadtman (*4*), boiled extract of *Cl.*

Kluyveri, EDTA and glutathione induced non-enzymatic oxidative reaction, which can be excluded in this experiment.

FIG. 2.



Composition: 0.4 ml. of 1 M phosphate buffer (pH 8.0), 30 μM/1.0 ml. of urocanic acid, 0.4 ml. of 10^{-2} M EDTA (final concentration 10^{-3} M) and 2.0 ml. of enzyme solution were mixed and the total amount was made up to 4.0 ml. with distilled water. The pH was adjusted to 8.0 by the addition of 4 per cent sodium hydroxide and shaken at 37.5°. Determination of succinic monoureide: 1.0 ml. of 30 per cent trichloroacetic acid was added and to 1.0 ml. of the deproteinized filtrate were added 0.5 ml. of 30 per cent hydrochloric acid and 0.5 ml. of Fearon's reagent (diacetylmonoxime) and the solution was heated in a boiling water bath for 20 minutes. After cooling three drops of 3 per cent potassium persulfate was added. The solution was kept at room temperature for 15 minutes and the total amount was made up to 5.0 ml. with distilled water. The yellow colour was electrophotometrically estimated, using Filter S_{47} and a 10 mm vessel.

Stability of Succinic Monoureide against the Extract of Pseudomonas aeruginosa—This experiment was carried out aerobically using Warburg's manometer. As shown in Table I, the added succinic monoureide remained unchanged and O_2-uptake, formation of CO_2, NH_3, and urea were not proved. Moreover, an adaptive enzyme to succinic monoureide was not formed by living cells.

SUMMARY

1. Succinic monourede was isolated as the oxidative decomposition product of urocanic acid in the presence of EDTA to the extract of *Pseudomonas aeruginosa.*

TABLE I

Decomposition of Succinic Monoureide by the Extract of Pseudomonas Aeruginosa

	No EDTA	EDTA (10^{-3} *M*) added
Succinic monoureide remained	6 μM	6 μM
Oxygen uptake	—	—
CO_2-evolution	—	—
NH_3-evolution	—	—
Urea formation	—	—

Succinic monoureide: 6 nM.

2. In spite of the complete disappear of urocanic acid, the yield of succinic monoureide is small. Other intermediary metabolites may therefore be expected.

3. Succinic monoureide is not further metabolized by the extract of *Pseudomonas aeruginosa*.

ADDENDUM

Recently 0.1 g. of succinic acid was isolated from 9.0 g. of urocanic acid as the another oxidative decomposition product. Hydantoine acrylic acid was proved to open the hydantoine ring spontaneously at pH 8.0 and give the Fearon's carbamyl reaction.

We are indebted to Dr. T. Matsukawa (Research-Institute of Takeda Pharmaceutical Co., Ltd.) for elementary analysis and we wish to thank Dainihon Pharmaceutical Co., Ltd. for a kind gift of L-Histidine.

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potassium phosphate buffer solution used at that time, the influence of NH_4^+ in the absence of K^+ was studied.

MATERIALS AND METHODS

Enzyme Preparation—This was obtained from dried cells of *B. coli* phenologenes, which had been grown on peptone-bouillon medium containing 0.02 per cent tyrosine at 37° for 18 hours. The cells were harvested by centrifugation; the pack of paste was washed with a physiological saline solution and then dried in vacuo. Two g. of dried cells were ground with an equal amount of quartz-sand, extracted with 100 ml. of distilled water in a refrigerator for 1 hour and centrifuged at 12000 r.p.m. The supernatant was used as the crude enzyme solution.

Determination of Phenol—The reaction was stopped by the addition of 1 ml. of trichloroacetic acid and 5 ml. of a deproteinized filtrate was extracted with 20 ml. (10 ml. first and then twice with 5 ml. portions) of petroleum ether. To the extract were added 5 ml. of 5 per cent Na_2CO_3 and 0.5 ml. of 33 per cent Folin's phenol reagent and the blue colour was determined photometrically using the filter S_{66} and a 10 mm.-cuvette.

Determination of Pyruvate—Pyruvate was assayed by the Friedemann-Haugen's method with 2,4-dinitrophenylhydrazine. (*8*)

Determination of Ammonium—Ammonium was determined by the Russell's method (*9*) using the Conway's apparatus. (*10*)

Incubation Condition—Studies were conducted under the following conditions, unless otherwise specified; 0.6 ml. of 0.1 *M* tris-(hydroxymethyl)-aminomethane-hydrochloride (THAM-HCl) buffer (pH 7.8), 0.6 ml. of 1 *M* KCl, 2 mg. of tyrosine and 1 ml. of enzyme solution were mixed and the total volume was made up to 6 ml. with distilled water. The solution was incubated at 37.5° for 60 minutes.

RESULTS

Formation of Phenol, Pyruvate and NH_3—The above-mentioned enzyme preparation were dialyzed against distilled water at O° until the Nessler's reaction became negative (about 20 to 30 hours), and the enzyme reaction was carried out with Thunberg's tube.

As shown in Fig. 1, phenol, pyruvate and NH_3 were produced in an equimolar ratio simultaneously. From this data, it is clear that phenol is directly produced from tyrosine.

Phenol was not formed from *p*-hydroxybenzoic acid and *p*-hydroxyphenylpyruvate by this enzyme, but phenol formation from the latter was demonstrated by the addition of glutamic acid. Accordingly, phenol was formed in this case from tyrosine, which was produced from *p*-hydroxyphenylpyruvate by transamination.

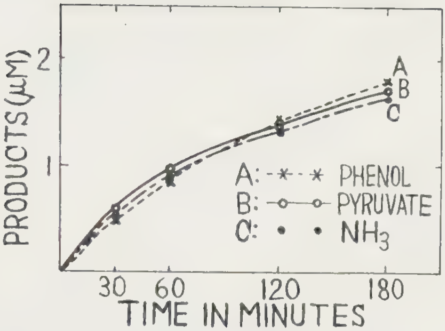

Fig. 1. Formation of phenol, pyruvate and NH_3.
0.6 ml. of 1 M potassium phosphate buffer and 10 μg. of pyridoxal phosphate were used.

The Effect of the Addition of Pyridoxal Phosphate—The dialyzed inactive enzyme regained its activity through the addition of pyridoxal phosphate as shown in Fig. 2. In this case the Michaelis constant K_m was close to 1.0×10^{-6} M. Pyridoxal + ATP was not able to replace pyridoxal phosphate as the cofactor of the enzyme. Therefore, the pyridoxalkinase appeared to have been inactivated in the preparation procedure.

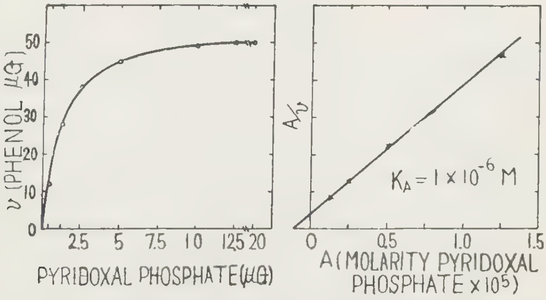

Fig. 2. The effect of the addition of pyridoxal phosphate.
0.6 ml. of 1 M potassium phosphate buffer was used. Incubation time, 30 minutes.

Influence of NH_4^+, K^+, Na^+ *and* Li^+—When THAM-HCl buffer was used instead of potassium phosphate buffer, the phenol formation decreased markedly (Fig. 3). It was increased by the addition of the NH_4^+ or K^+ to the solution. Fig. 3 shows the velocity concentration curves and the plots of A/v versus A for these ions.

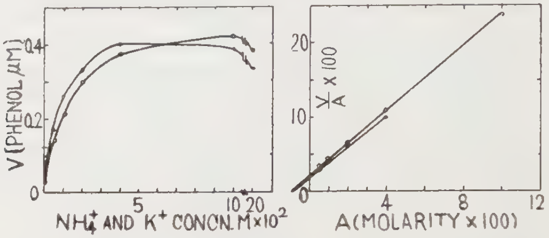

FIG. 3. The Activation of β-tyrosinase by NH_4^+ and K^+.
0.6 ml. of 0.1 *M* THAM-HCl buffer and 2 μg. of pyridoxal phosphate and 1 ml. of the dialyzed enzyme were used. Incubation time, 30 minutes. —●—●— NH_4^+, —○—○— K^+.
Km(NH_4^+)=0.0092 *M*, Km(K^+)=0.0098 *M*.

During the process of the minimal phenol formation by the endogenous NH_4^+ in the enzyme preparation, the phenol formation increased markedly with the addition of NH_4^+ (Fig. 4).

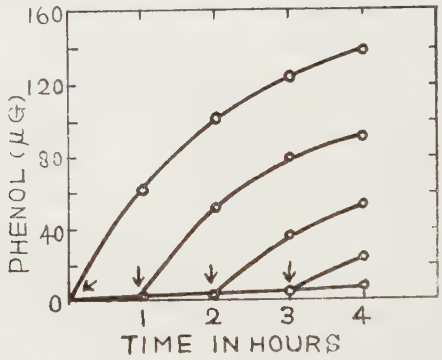

FIG. 4. The effect of NH_4^+ and incubation time.
The arrow addition of 0.15 ml. of 1 *M* $(NH_4)_2SO_4$. 2 μg. of pyridoxal phosphate and 1 ml. of the dialyzed enzyme solution were used.

On the contrary, Na^+ and Li^+ inhibited the enzyme action activated by K^+ and NH_4^+, as shown in Fig. 5.

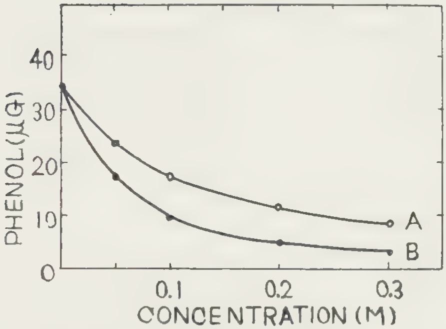

FIG. 5. The inhibition of K^+ activation of β-tyrosinase by Li^+ and Na^+. THAM buffer, enzyme and Na^+ or Li^+ were first incubated at O° for 30 min.: then water, K^+, pyridoxal phosphate (2 μg) and tyrosine were added and the tubes incubated at 37.5° for 30 minutes. Curve A; Na^+ as NaCl, Curve B; Li^+ as Li_2SO_4. 1 ml. of the dialyzed enzyme was used.

Inhibitors—As shown in Tables I and II, the metal chelating compounds and SH-reagents inhibited the activity of β-tyrosinase. The activity of the enzyme, inhibited by PCMB, was restored by the addition of mercapto compounds, *e.g.*, glutathione and cysteine (Fig. 6).

DISCUSSION

As described above, it was confirmed that phenol, pyruvate and NH_3 were produced from tyrosine in an equimolar ratio simultaneously. Consequently, it is apparent, that β-tyrosinase catalyzes the primary fission of the side chain of tyrosine at the β-carbon position, as reported in the previous paper. (*3*, *4*) It was also confirmed that pyridoxal phosphate is the coenzyme of β-tyrosinase and the dissociation constant of the pyridoxal phosphate-β-tyrosinase complex ($K=1.0\times10^{-6}$ mole per liter) approximates that of tryptophanase ($K=2.1\times10^{-6}$ mole per liter) (*11*) and glutamic aspartic transaminase ($K=1.5\times10^{-6}$ mole per liter) (*12*).

In the above experiment, using THAM buffer, K^+ and NH_4^+ activated phenol formation, as the case of indole formation. The negative result of NH_4^+ obtained in the previous report (*4*), therefore, must be attributed to the potassium phosphate buffer used then.

The Michaelis constant for these ions have been found to closely

TABLE I

The Inhibition of β-Tyrosinase by Metal-chelating Reagents

Inhibitor	Concentration (M)	Phenol (μg.)	Inhibition (%)
—	—	50	—
o-Phenanthroline	10^{-3} 2×10^{-3}	8 0	84 100
Nitroso-R-salt	10^{-3} 5×10^{-3}	18 3	64 94
α,α′-Dipyridyl	10^{-3} 5×10^{-3}	30 19	40 62
Oxine	10^{-3} 5×10^{-3}	38 26	24 48
Sodium diethyldithiocarbaminate	10^{-3} 5×10^{-3}	28 17	44 64
EDTA	10^{-3} 10^{-2}	44 24	12 52
Azide	10^{-3} 5×10^{-3}	50 40	0 20
KCN*	10^{-3}	10	80
NH_2OH*	10^{-3}	0	100

*: These compounds also react with the carbonyl group.

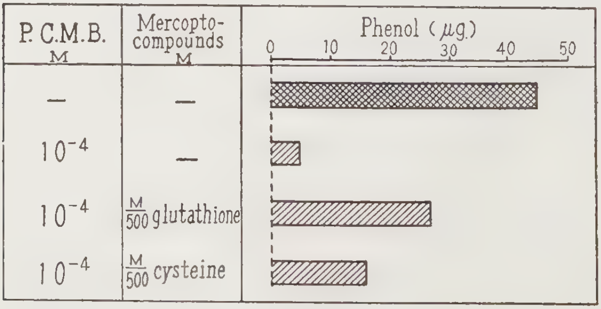

FIG. 6. The influence of SH-reagents and mercapto-compounds.

TABLE II

The Inhibition of β-Tyrosinase by SH-Reagents

Inhibitor	Concentration (M)	Phenol formation (μg.)	Inhibition (%)
—		50	
PCMB	10^{-4}	7	86
”	2×10^{-4}	3	94
Monoiodoacetate	10^{-3}	50	0
”	2×10^{-3}	43	14
”	10^{-2}	24	53
Ferricyanide	10^{-3}	22	56
Cu^{++}	10^{-3}	20	60
Hg^{++}	10^{-3}	0	100

resemble one another. This facts suggest an equivalence of the reactive mechanism of both ions. The counteraction of Na^+ and Li^+ for K^+ and NH_4^+ activation was proved. The kinetics between these ions and the enzyme reaction will be discussed in the following papers (indole formation).

From the attitudes of the metal catchers, the participation of the heavy metals was strongly suggested. The activity of the enzyme inhibited with *o*-phenanthroline was actually partially restored by the addition of a certain heavy metal. Enzyme purification is required before reaching a reliable conclusion.

As the enzyme action was inhibited by the SH-reagents and restored by the addition of glutathione and cysteine (Fig. 6), the SH-group of the apo-part is important for the reaction.

SUMMARY

1. Phenol, pyruvate and NH_3 are produced from tyrosine by the β-tyrosinase, obtained from B. coli phenologenes, in an equimolar ratio simultaneously.

2. Pyridoxal phosphate is the coenzyme of the β-tyrosinase.

3. For this reaction, the presence of NH_4^+ or K^+ is required and Na^+ or Li^+ counteract K^+ or NH_4^+ activation. The previous report was thus corrected.

4. This enzyme is inhibited by metal chelating compounds. Therefore, the participation of a certain heavy metal is suggested.

5. This enzyme is also inhibited by various SH reagents, and regains its activity by the addition of mercapto compounds. Concequently the participation of the SH group is suggested.

6. The mechanism of the enzymatic reaction is discussed.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

CONSECUTIVE HYDROLYSIS OF PHOSPHODIESTER BY PHOSPHODIESTERASE AND PHOSPHOMONOESTERASE

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(Received for publication, August 3, 1956)

Consecutive hydrolysis of a substrate ABC which proceeds in the reaction sequence ABC→AB+C→A+B+C, was previously investigated using diglycyl diaminobenzoate as the substrate (*1*). The present communication is the result of studies on the kinetics of the consecutive hydrolysis of ethyl *p*-nitrophenylphosphate (NPPE) hydrolysable at its optimum pH 9 by the diesterase of snake (Habu) venom into *p*-nitrophenol and ethyl phosphate (*2*). Urine monoesterase hydrolyzes ethyl phosphate at its optimum pH 5. Therefore, in case the diesterase and monoesterase act together upon NPPE at pH 7 the reaction proceeds in two steps and the amount of *p*-nirophenol liberated (at time intervals) would be equivalent to that of NPPE hydrolyzed, and hence to the sum of the amounts of ethyl phosphate remaining at the moment and lost by successive hydrolysis, whereas the amount of inorganic phosphate estimated in the same aliquot would correspond to the amount of ethyl phosphate hydrolyzed. Hence, ethyl phosphate remaining at any time is measurable as the difference of the amounts of *p*-nitrophenol and the free inorganic phosphate liberated. Theoretical pursuit of the process of this consecutive reaction seems not to be simple, since the competitive inhibition of the diesterase activity by ethyl phosphate and inorganic phosphate as well as that of the monoesterase activity by NPPE and inorganic phosphate would complicate the reaction process. For the analysis of this reaction, the enzyme-inhibitor dissociation constants have been estimated. Some methods for the estimation of these constants are presented in this paper and, taking those inhibitory effects into consideration, the course of increase and decrease in ethyl phosphate is discussed.

METHODS

Calcium ethyl *p*-nitrophenylphosphate was prepared according to the method of Nishibori (*2*). Diesterase solution of snake (Habu) venom was made free from a trace of monoesterase by adsorption on caolin (*3*). As the monoesterase a human urine sample concentrated and dialyzed was used. *p*-Nitrophenol liberated was estimated after deproteinizing an aliquot of the test solution with 5 per cent sulfosalicylic acid and alkalizing the filtrate with by sodium carbonate solution. Estimation of inorganic phosphate for measuring the initial rate of hydrolysis of ethyl phosphate or inorganic pyrophosphate was made by the safranine method (*4*). Phenol liberation from phenylphosphate was estimated by indophenol method (*4*). These micromethods were adopted because an increase in products proportional to the time should be realized within the range not exceeding far over 10 per cent hydrolysis of the substrate. However, to pursue the whole course of inorganic phosphate production, the Fiske-Subbarow's method was used. The test was carried out at 37° in the veronal buffer.

RESULTS

pK_m of Diesterase-NPPE Complex—Ethyl *p*-nitrophenylphosphate solutions in serial concentrations from $M/100$ to $M/400$ were respectively hydrolyzed by the diesterase and the initial rate of *p*-nitrophenol liberation was measured. pK_m calculated graphically according to the equations derived by Lineweaver and Burk (*5*) gave 1.75.

pK_i of Diesterase-Ethyl Phosphate Complex—The initial rate of hydrolysis of NPPE of $M/1000$ concentration was compared with those in the presence of ethyl phosphate in concentrations from $M/2000$ to $M/250$ and the diesterase inhibitor dissociation constant pK_i obtained from the following equation 1 of Michaelis-Menten (*6*) was 3.21.

$$K_i = \frac{\mathrm{I} \times K_m}{(S+K_m)\,(V_o/V-\mathrm{I})} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (1)$$

pK_i of Diesterase-Inorganic Phosphate Complex—The experiment was carried out similarly just as in the above case with the addition of inorganic phosphate at $M/2000$ to $M/500$ concentrations. The pK_i found was 3.23, coinciding unexpectedly with that of diesterase-ethyl phosphate complex.

pK_m of Monoesterase-Ethyl Phosphate—This was estimated directly by measuring the initial velocity of hydrolysis of ethyl phosphate of various concentrations. pK_m was 2.49. It was also possible to determine indirectly from the inhibiting action of ethyl phosphate to phenylphosphate hydrolysis as follows, since pK_m of monoesterase-phenylphosphate compound has been beforehand estimated to be 3.08. Phenol liberation at initial stages from $M/1000$ phenyl phsophate solution in the presence of ethyl phosphate of $M/1000$ to $M/200$ concentrations was measured by the dibromoaminophenol method (*4*). pK_i of ethyl phosphate calcu-

lated according to the Eq. 1 of Michaelis-Menten was 2.55 and agreed with pK_m of monoesterase-ethyl phosphate complex, found to be 2.49 by the direct method.

pK_i of Monoesterase-NPPE Complex—Hydrolysis of ethyl phosphate of $M/1000$ concentration was measured at is initial stages in the presence of NPPE in concentrations from $M/2000$ to $M/500$. Inorganic phosphate liberated was estimated. Calculation according to Michaelis and Menten gave pK_i of 1.87.

pK_i of Monoesterase-Inorganic Phosphate complex—An exact measurement of ethyl phosphate hydrolysis is difficult to be carried out in the presence of inorganic phosphate added. Therefore, three independent methods were tested which gave the pK_i values coinciding fairly well with each other.

The first is based on the inhibition of inorganic phosphate to the phenyl phosphate hydrolysis by the monoesterase. Phenol liberation at initial stages in $M/1000$ substrate solution in the presence of inorganic phosphate of $M/4000$, $M/2000$, and $M/1000$ concentrations was measured by the dibromoaminophenol method. Since pK_m of monoesterase-phenylphosphate compound is known to be 3.08, pK_i of inorganic phosphate could be calculated as usual, to give 3.70.

The second method refers to the analysis of the course of ethyl phosphate hydrolysis by urine monoesterase. The progress of the reaction should obey the following equation 2 of Michaelis-Menten where a is the concentration of ethyl phosphate at the start and x is that hydrolyzed at time t.

$$\frac{a}{t}\left(\frac{K_m}{a}+\frac{K_m}{K_i}\right)\ln\frac{a}{a-x}+\frac{x}{t}\left(1-\frac{K_m}{K_i}\right)=k \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (2)$$

$$\frac{1}{t}\left(\frac{K_m}{a}+\frac{K_m}{K_i}\right)\log\frac{a}{a-x}+0.4343\frac{x}{at}\left(1-\frac{K_m}{K_i}\right)=k'$$

To obtain K_i, this equation was modified as follows:

$$\left(\frac{K_m}{a}+\frac{K_m}{K_i}\right)X+\left(1-\frac{K_m}{K_i}\right)Y=k',$$

hence

$$Y=k'-\left(\frac{K_m}{a}+\frac{K_m}{K_i}\right)X \Big/ \left(1-\frac{K_m}{K_i}\right)$$

$$\tan\theta=-\left(\frac{K_m}{a}+\frac{K_m}{K_i}\right) \Big/ \left(1-\frac{K_m}{K_i}\right)$$

When Y is plotted against X, K_i will be obtained from the tangent of the straight line drawn through the experimental points, since K_m is already known. The experiment was carried out with $M/1000$ ethyl phosphate solution (Table I and Fig. 1). pK_i found was 3.63, agreeing well with 3.70 stated above.

TABLE I

Hydrolysis of NPPE and Ethyl Phosphate

Substrate	NPPE			Ethyl phosphate		
Enzyme	Phosphodiesterase			Phosphomonoesterase		
t (*hours*)	Hydrolysis (%)	X	Y	Hydrolysis (%)	X'	Y'
0.25	6.5	0.117	0.113	6.7	0.120	0.116
0.5	12.5	0.116	0.109	12.0	0.111	0.104
1.0	22.4	0.110	0.097	18.9	0.091	0.082
2.0	36.4	0.098	0.079	32.7	0.086	0.071
2.5	41.3	0.093	0·072	38.4	0.084	0.067
3.0	47.0	0.092	0.068	41.4	0.077	0.061
3.5				45.3	0.075	0.056
4.0	53.2	0.083	0.068	47.5	0.070	0.052

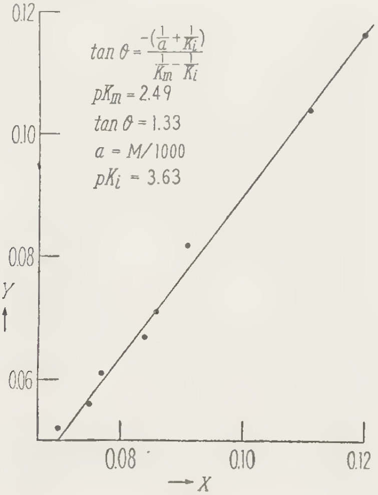
Experimental conditions: 6 ml. of $M/200$ substrate solution and 12 ml. of water were preincubated at 37° and to the mixture was added another similarly preincubated mixture of 6 ml. of the respective enzyme solution and 6 ml. of veronal buffer. The final concentration of each substrate was $M/1000$, pH. 7. For X, Y, X' and Y', see text.

The third method is similar in principle to the second except that phenyl phosphate is used as the substrate. Since pK_m 3.08 of monoesterase phenyl phosphate is known, pK_i was calculated and found to be 3.80.

In a similar manner, it was possible to calculate pK_i of diesterase-ethyl phosphate compound by the analysis of the course of NPPE hydrolysis. The concentration of NPPE was $M/1000$ and the *p*-nitrophenol liberation was measured. The results are presented in Table I and Fig. 2. The pK_i found was 3.23, agreeing well with 3.21, mentioned above in the case of inhibition by ethyl phosphate of NPPE hydrolysis.

This procedure can by applied to other enzymes, for example to

Fig. 1. Estimation of pK_m by analysis of the course of ethyl phosphate hydrolysis.



(See text and Table I for details.)

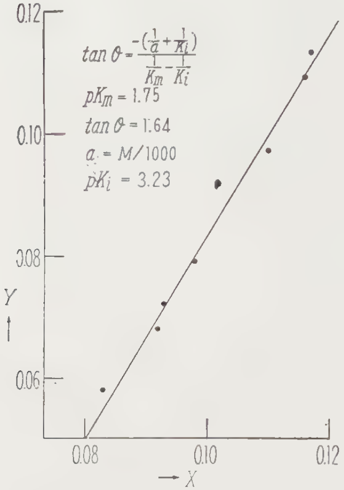
inorganic pyrophosphatase. In this case the Eq. 2 of Michaelis-Menten must be slightly modified; $1/K_i$ of the original formula is substituted by $2/K_i$, since two moles of inorganic phosphate are produced from one mole of pyrophosphate. After previously estimating pKm as usual of pyrophosphate compound with yeast pyrophosphatase to be 3.30, the progress of the enzyme action was pursued as far as above 50 per cent hydrolysis and the pKm of inorganic phosphate-pyrophosphatase complex was graphically measured. It was 3.34.

The negative logarithms of the dissociation constants of the diesterase or monoesterase compounds with the respective substrates or their hydrolytic products are summarized in Table II.

Consecutive Hydrolysis of Ethylester of p-Nitrophenylphosphate (*NPPE*)—The enzyme solutions applied for this test had the activities shown in Table III. Ratio of the activities was 1 :0.55.

Hydrolysis of NPPE by the mixture of the diesterase and monoesterase solutions (1 :1) was measured up to nearly 100 per cent production of free *p*-nitrophenol, whereby inorganic phosphate liberated was estimated in the same aliquot. The values thus obtained indicate as described in the introduction of this paper, the progess of the NPPE

FIG. 2. Estimation of pKm by analysis of the course of NPPE hydrolysis.



(See text and Table I for details.)

disappearance, the hydrolysis of ethyl phosphate intermediately produced, and the increase and successive decrease in this intermediate exsisting in the reaction medium. The results are shown in Table IV and Fig. 3. The maximum amount of ethyl phosphate accumulated was 46.8 per cent of NPPE used for the test.

DISCUSSION

In studies on the kinetics of phosphatase the affinities of the substrate as well as phosphate compounds produced should be taken into consideration. Inhibition by hydroxy compounds such as *p*-nitrophenol or ethanol are negligible so long as their concentration is low, as in the present experiment.

According to the Eq. 2 of Michaelis and Menten shown above, any enzymatic reaction, in which one mole of an inhibitory substance is produced from one mole of the substrate, would be monomolecular only where K_m and K_i are equal. In the case of hydrolysis of ethyl *p*-nitrophenylphosphate (NPPE) by single action of phosphodiesterase, it was found that the reaction is not monomolecular and proceeds

Table II

pK_m and pK_i of Phosphate Compounds with Diesterase or Monoesterase

Enzyme	Phosphate compound	pK_m	pK_i	Estimated from
Venom diesterase	NPPE	1.75		initial rates of hydrolysis.
	Ethyl phosphate		3.21	inhibition of NPPE hydrolysis by ethyl phosphate added.
	”		3.23	analysis of curve of NPPE hydrolysis to the extent over 50%.
	Inorganic phosphate		3.23	Inhibition of NPPE hydrolysis by inorganic phosphate added.
Urine monoesterase	Ethyl phosphate	2.49		initial rates of hydrolysis.
	”	2.55		inhibition of phenylphosphate hydrolysis by ethyl phosphate added.
	Inorganic phosphate		3.70	inhibition of phenyl phosphate hydrolysis by inorganic phosphate added.
	”		3.63	analysis of the curve of ethyl phosphate hydrolysis.
	”		3.80	analysis of the curve of phenyl phosphate hydrolysis.
	NPPE		1.87	inhibition of ethyl phosphate hydrolysis by NPPE added.

Table III

The Ratio of the Activites of Diesterase and Monoesterase

Substrate	Enzyme	Hydrolysis in		Ratio of initial velocities
		2 minutes	4 minutes	
NPPE	Venom diesterase	5.6%	10.9%	1:0.55
Ethyl phosphate	Urine monoesterase	3.1%	6.0%	

Experimental conditions: *cf.* the undernote of Table I.

obeying that Eq. 2. The same equation can be applied for the consecutive hydrolysis of NPPE by the combined action of diesterase and monoesterase, since pK_i of diesterase-ethyl phosphate compound is known

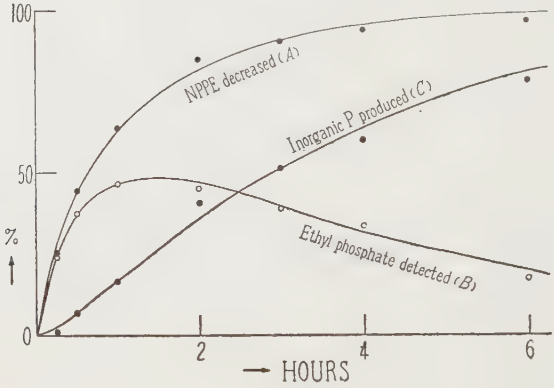
TABLE IV

Consecutive Hydrolysis of NPPE

Reaction time *hours*	0.25	0.5	1.0	2.0	3.0	4.0	6.0
NPPE decreased %	25.5	44.6	63.8	85.5	90.5	93.8	96.0
Inorganic P produced %	1.3	7.3	17.0	40.6	51.7	60.0	78.1
Ethyl phosphate present %	24.2	37.3	46.8	44.9	38.8	33.8	17.9

The final concentration of NPPE was $M/1000$. A mixture of the diesterase and monoesterase solutions (1:1) was used. Ratio of the activities was 1:0.55 (see text and Table III). Experimental conditions were the same as those in Table I.

FIG. 3. Consecutive hydrolysis of NPPE.



(See Table III and text for details.)

to be the same with that of diesterase inorganic phosphate. The reaction constant of the equation 2 can be obtained by slightly modifying the formula in the following manner and plotting t against X.

$$t=X/k, \quad \text{where}$$

$$X=K_m\left(1+\frac{a}{K_i}\right) 2.303 \log \frac{a}{a-x} + x\left(1-\frac{K_m}{K_i}\right)$$

The cotangent of the line drawn from the origin of coordinate system through the points calculated from the data of Table IV gives the average of k. Once this constant is known, a theoretical course of

NPPE hydrolysis can be traced in a simple manner by calculating t corresponding to any x values chosen at random. It is illustrated as curve *A* in Fig. 3. The points indicating the experimental values are situated on this theoretical curve.

Though the hydrolysis of ethyl phosphate itself also obeys the Eq. 2, it must be considered in the consecutive hydrolysis of NPPE, that NPPE may interfere with the monoesterase action on ethyl phosphate. However, the inhibitory effect of NPPE to monoesterase can be neglected, because pK_i of monoesterase-NPPE complex is smaller by nearly 0.7 unit than the pK_m of monoesterase-ethyl phosphate and in the present case the concentration of NPPE is $M/1000$ (pS 3), so low that even at the start of the reaction no more than a few per cent of the total monoesterase would be bound by NPPE.

Now it is known that if each step of a consecutive reaction, $ABC \rightarrow AB + C \rightarrow A + B + C$, is monomolecular, the amounts of AB and A at time t will be given respectively by the following equations, where k_1 and k_2 are velocity constants of respective monomolecular reactions.

$$(AB)_t = (ABC)_0 \frac{k_1}{k_2-k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \quad \text{...............} \quad (3)$$

$$(A)_t = (ABC)_0 \left(1 - \frac{k_2}{k_2-k_1} e^{-k_1 t} + \frac{k_1}{k_2-k_1} e^{-k_2 t}\right) \quad \text{...............} \quad (4)$$

However, in the present case the fact is that both reaction steps are not monomolecular, and the data in Table I indicate that the values X and X', which should be the reaction constants if both the reactions were monomolecular, decay gradually with time. Therefore, a mathematical treatment for finding $(AB)_t$ and $(A)_t$ seems very difficult. Fortunately, the ratio of those X and X' values found at each corresponding time t was found to remain nearly the same throughout the course of the reaction. Hence, the following consideration was made to simplify the matter.

Assuming that if each amount x, x' and so on of NPPE were hydrolyzed at time t, t'...owing to the independent monomolecular reactions proceeding with respective k_1, k_1'..., then k_2 k_2'...at t, t'...of the second reaction, namely, the hydrolysis of ethyl phosphate, would be $0.55 \times (k_1$ or k_1'...), since the ratio of activities of diesterase and monoesterase applied for the consecutive hydrolysis of NPPE was 1:0.55. Any point situated on the theoretical curve *A* (Fig. 3) indicated above of NPPE hydrolysis would give by calculation one of those k_1 values, and hence further the corresponding k_2 values.

By introducing each pair of k_1 and k_2 values at respective time t to the above Eqs. 3 and 4, it was possible to draw the other theoretical curves B and C (Fig. 3) illustrating the increase and decrease of ethyl phosphate and the production of inorganic phosphate, respectively. The points indicating the experimental data are situated on these theoretical curves. The maximum amount of ethyl phosphate existing in the medium can also be calculated from the ratio $k_1:k_2=1:0.55$. It is found to be 48 per cent and in fact to be seen in Fig. 3.

SUMMARY

The phosphodiesterase of snake (Habu) venom partly purified free from monoesterase liberates *p*-nitrophenol from ethyl ester of *p*-nitrophenyl phosphoric acid. Ethyl phosphate itself is hydrolyzable by urine monoesterase. Combined action of those two enzymes produces ethyl phosphate as an intermediate, the amount of which increases to a maximum extent and then decreases. The form of curves indicating the decrease of the substrate, the increase and successive decrease of ethyl phosphate, and the progressive increase of free inorganic phosphate, respectively, were discussed, taking into consideration the affinities of the substrate and its hydrolytic products to each of the two enzymes. The experimental data agreed fairly well with the values calculated applying those affinity constants and the activities of the enzyme solutions applied.

Some methods for the estimation of the dissociation constants of enzyme-inhibitor compounds were tested with satisfactory results.

The work was aided by a Grant in Aid of Scientific Research from the Ministry of Education.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

ON THE OPENING AND SPLITTING OF INDOLE OF TRYPTOPHAN*

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(Received for publication, August 3, 1956)

From the experiments on the conversion of substituted derivatives of tryptophan to the corresponding indole derivatives, Ichihara *et al.* (*1*) postulated that the hydrogen in the pyrrole ring of the tryptophan molecules is strictly required for this enzyme system, but the hydrogen in the benzene part of the molecules may be substituted by some other group. It was also discussed to be likely that the tryptophan peroxidase-oxidase system may require same specificity on the pyrrole part of the tryptophan molecules.

However, Hayaishi and Stanier (*2*) previously demonstrated that 5 and 7-hydroxytryptophans are not converted to the corresponding kynurenine derivatives by cell-free extracts of Pseudomonas strain 23, and that these compounds competitively inhibit the enzymatic oxidation of L-tryptophan. Moreover 2-hydroxy, and 4-, 5-, and 7-methyl-tryptophans are not attacked by this enzyme, and similar experiments with 5- and 7-hydroxytryptophans conducted by Mehler (*3*) with the animal enzyme have also shown negative results. It was, therefore, necessary to make more experiments in the indole formation from other substituted derivatives of tryptophan.

Yonezawa isolated unexpectedly a strain from the air in our laboratory in the summer season which cleavages indole and tryptophan, but can not scatole. The present investigation was undertaken to examine the possible cleavage of the substituted derivatives of indole and pyrrole by this bacteria in order to study the mechanism of the cleavage of the indole ring.

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* This work was supported by a grant for scientific research from the Ministry of Education. We should like to express our hearty gratitude.

EXPERIMENTAL

Materials

5-Methylindole and 1-Methylindole—Synthesized after Robson (*4*) and Matsui *et al.* (*5*) respectively.

7-Hydroxyindole and 5-Hydroxytryptophan—Furnished by Dr. Hayaishi.

5-Methyltryptophan—Preparation of Kahlbaum Pharmaceutical Co.

2-Methyl-3-Carbethoxypyrrole and 2-Methyl-3-Carboxypyrrole—Synthesized after Akabori. (*6*)

Indole Decomposing Strain (*Y-Strain*)—Yonezawa (1944) isolated a strain from the air of our laboratory in summer, which decomposes indole and tryptophan but does not scatole. For convenience this strain is called the Y-strain. The Y-strain was cultivated in the following culture medium, containing 200 mg./l. of the indole derivatives. After 18–20 hours the cells were harvested. The cells were washed with physiological saline solution until the wash water gave no longer the indole reaction with Ehrlich's aldehyde reagent and resuspended in *M*/10 phosphate buffer (pH 7.4) in a ratio of 10 mg./ml.

Composition of the culture medium; 10 g. of yeast, 1.0 gm of glucose, 5 g. of K_2HPO_4 and 1 l. of water. The mixture was heated at 100° and the filtrate was used. The pH was 7.4.

Indole forming Strain—*E. coli* K-12 was cultivated in bouillon and harvested after 18–20 hours. The cells were washed and dried as described above. The dried cells were ground with an equal amount of sea sand and extracted with distilled water in a ratio of 20 mg./ml. in a refrigerator for one hour. The supernatant was used as the enzyme solution.

Liver Extract of the Rabbit—The rabbit was sacrificed by bleeding and the liver was ground with an equal amount of sea sand. The brei was extracted with three times the volume of *M*/10 phosphate buffer (pH 7.4) in a refrigerator for one hour and centrifuged. The supernatant was used as the crude enzyme solution.

Methods

Determination of Indole Derivatives After incubation, 1.0 ml. of 30 per cent trichloroacetic acid was added and the mixture extracted twice with 5 ml. portions of petroleum ether. To the etherial layer was added 5.0 ml. of Ehrlich's aldehyde reagent, composed of 4.0 g. of *p*-dimethylaminobenzaldehyde, 80 ml. of 30 per cent HCl and 380 ml. of 90 per cent alcohol. The colour intensity was electrophotometrically determined, using Filter S_{53} for indole, 1-methylindole and 5-methylindole and S_{57} for 7-hydroxyindole and a 10 mm.-vessel. By this method $0 \sim 200\ \gamma$ of substrate can be estimated.

Determination of Pyrrole Derivatives—After incubation, 1.0 ml. of 30 per cent trichloroacetic acid was added. The tubid solution was extracted twice with 5 ml. portions of ether. The etherial solution was added with 5.0 ml. of Ehrlich's reagent and the intensity of the colour developed was electrophotometrically estimated, using the Filter S_{53} and a 10 mm.-vessel.

Determination of Tryptophan Derivatives—After incubation, 1.0 ml. of 30 per cent trichloroacetic acid was added, and 1.0 ml. of the deproteinized filtrate was used for the estimation of 5-hydroxy- and 5-methyl-tryptophan according to the method of Mason and Berg, modified by Kawamata (*7*).

Condition of Reaction—This is as follows:

Substrate (amount is shown in tables)	1.0 ml.
1 *M* Phosphate Buffer (pH 7.4)	0.4 ml.
(for the indole formation: pH 8.0)	
Enzyme solution or cell suspension	1.0 ml.

Total volume was 4.0 ml. made up with distilled water and incubated at 37.5° for 1∼2.5 hours.

RESULTS

A. Indole Formation from Tryptophan Derivatives by the Extract of E. coli—Substituted indole is formed from 5-methyltryptophan, but not from 5-hydroxytryptophan. Furthermore 5-hydroxytryptophan does not inhibit the formation of indole from tryptophan. (TableI)

TABLE I

Indole Formation from Tryptophan Derivatives by the Extract of E. coli

Substrate	Enzyme solution	pH	Indole formed
5-Hydroxytryptophan (10 μM)	1.0 *ml.* 1.0	6.8 8.0	0 γ 0
5-Methyltryptophan (10 μM)	1.0 1.0	6.8 8.0	10 13
Tryptophan (10 μM)	1.0 1.0	6.8 8.0	30 35
Tryptophan (10 μM)+5-hydroxytryptophan (10 μM)	1.0 1.0	6.8 8.0	30 35

B. Decomposition of Indole and Pyrrole Derivatives by the Y-Strain—The metabolic pathway of indole by the Y-strain has been established as follows (*8*): Indole → (indoxyl) → (dihydroxyindole) → isatin → formlyanthranilic acid → anthranilic acid → salicylic acid → catechol. The first step in the decomposition takes place at the pyrrole moiety of the indole ring. As shown in Table II, 5-methyl- and 7-hydroxylindoles and indole were completely or almost completely decomposed, while 1-methylindole and three pyrrole derivatives remained unchanged. Without addition of the enzyme 7-hydroxylindole is not decomposed (see Table II). The decomposition of indole is not inhibited by 5-hydroxytryptophan as found in the case of tryptophan (Hayaishi *et al.* and in Table III).

TABLE II

Decomposition of Indole- and Pyrrole-derivatives by the Suspension of the Υ-Strain

Substrate	Amount added	Reaction time	pH	Indole remaining	Notice
	γ	*hrs.*		γ	
5-Methylindole	50 100	2.5 2.5	7.4 7.4	0 0	
7-Hydroxyindole	66.5 66.5	1.0 1.0	6.8 8.0	1.6 3.2	Kept in $M/10$ phosphate buf- (pH 6.8 and 8.0) at $37_{\circ}$ for 7 days without change
1-Methylindole	131	2.5	7.4	131	
2-Methyl-3-carbethoxy-pyrrole	80	2.0	7.4	80	
2-Methyl-3-carboxy-pyrrole	80	2.0	7.4	80	
Pyrrole	80	2.0	7.4	80	
Indole	100	1.0	7.4	0	
Indole (58.5 γ + 5-hydroxytryptophan (110 γ)	58.5 58.5	1.0 1.0	6.8 8.0	0 0.2	

C. Decomposition of Tryptophan Derivatives—As shown in Table III, tryptophan and 5-methyltryptophan are decomposed by the liver extract, while 5-hydroxytryptophan is not only unchanged but inhibits the decomposition of tryptophan, as reported by Hayaishi *et al.* The decomposition of 5-methyltryptophan is much less than that of tryptophan.

TABLE III

Decomposition of Tryptophan Derivatives by the Liver Extract

Substrate	Enzyme solution	Kynurenine γ
5-Hydroxytryptophan (2 μM)	Liver extract	0
	Y-strain cell suspension	0
5-Methyltryptophan (2 μM)	Liver extract	20
	Y-strain cell suspension	34
Tryptophan (2 μM)	Liver extract	135
Tryptophan (2 μM)+5-hydroxy-tryptyphan (2 μM)	Liver extract	15

37°, 3 hours.

* Kynurenine derivative is calculated as kynurenine.

DISCUSSION

As Happold (*9*) reported, 5-methyltryptophan is converted to indole by the extract of *E. coli*, though to much lesser extent compared to tryptophan, 5-hydroxytryptophan is not attacked by this enzyme, and did not inhibit the formation of indole from tryptophan. (Table I)

The interesting results were obtained about the cleavage of the indole ring. 5-hydroxytryptophan was not attacked by liver extracts and Y-Strain and had the inhibitory effect on the oxidation of tryptophan, as Hayaishi described. However the situation was somewhat different with 5-methyltryptophan as a substrate, and this compound was shown to be cleaved at an appreciable rate by liver extracts or by Y-strain (Table III), in contrast to the findings with Pseudomonas.

The cleavage of indole- and pyrrole-derivatives by Y-strain was very different from the results with tryptophans. This organism cleaved 5-methylindole as had been anticipated but not 1-methylindole. However 7-hydroxyindole was attacked almost completely by this bacterium, and 5-hydroxytryptophan did not inhibit the cleavage of indole (Table II). On the contrary, three other pyrrole compounds tested were not cleaved, in spite of the presence of hydrogen atom in the 1- and 5-position (2′) of the pyrrole molecule (Table II). These data seem to suggest a possibility that the benzene part combined to the pyrrole ring has the important significance to the cleavage of the pyrrole moiety of indole ring.

Recently Bonner (*10*) obtained one mutant of *Neurospora* which required 7-hydroxytryptophan for growth, but did not respond to tryptophan. These data will suggest that 3-hydroxykynurenine is produced directly. Thus our assumption (*1*), that the tryptophan-derivatives substituted in the benzene ring may be cleaved, in contrast to those substituted in the pyrrole ring, was shown to be correct, thought the limit became narrow.

SUMMARY

1. From 5-Hydroxytryptophan the substituted indole was not formed by the extract of *E. coli* but did not inhibit indole formation from tryptophan. The substituted indole was formed from 5-methyltryptophan.

2. 5-Methyltryptophan was decomposed by the liver extract of rabbit and the cell suspension of Y-strain and gave a kynurenine reaction, but 5-hydroxytryptophan was not decomposed and rather inhibited the kynurenine formation from tryptophan.

3. 5-Methylindole and 7-hydroxyindole were decomposed completely by the Y-strain. Pyrrole, 2-methyl-3-carbethoxypyrrole, 2-methyl-3-carboxypyrrole and 1-methylindole were not decomposed. The benzene nucleus of indole must be important for the opening of pyrrole ring. It was suggested, that the benzene moiety of indole can not be cleaved before the pyrrole ring opens.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

BIOCHEMICAL STUDIES ON STREPTOLYSIN S.

I. EFFECTS OF ALKALI-TREATED RIBONUCLEIC ACID IN THE HEMOLYSIN FORMATION

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(Received for publication, August 6, 1956)

In 1939, it was demonstrated by Okamoto (*1*) that the formation of the hemolysin by hemolytic *Streptococci* is greatly enhanced by the addition of nucleic acid to the culture medium. This nucleic-acid effect on the hemolysin formation was confirmed by many workers (*2 4*) and expedited the progress in the study of the hemolysin. The research on this effect was greatly simplified owing to the fact that the enhancement of hemolysin formation is also observed by suspending resting cells in nucleic acid solution for limited periods of time (*5*). This observation was also comfirmed by Bernheimer (*6*).

Although the nature of nucleic acid which enhances the hemolysin formation has been studied (*2–8*), the basic properties of ribonucleic acid (RNA) which is required for such effect are still obscure. It is the purpose of this study to reinvestigate the relationships between the nature of RNA and its hemolysin-forming potency.

As for the alkaline treatment, it was found that complete hydrolysis of RNA deprives it of the hemolysin-forming potency and RNA treated with alkali in the course of its preparation has little potency. But there is no further information as to the RNA treated with alkali in various degrees. In this paper attention was paid to the reinvestigation of the potency of nucleic acid which was hydrolyzed to various degrees with mild alkali. The information so obtained could then be compared with the results after other types of hydrolysis.

EXPERIMENTAL

I. Determination of the Potency

Cell Suspension—Cells of the S. 8 strain of hemolytic *Streptococci*, cultivated in 100 ml. of ordinary broth medium for 8.5 hours, were collected by centrifugation. The sedimented cells were washed with phosphate-buffered saline ($M/30$ at pH 7.0 and $M/12.9$ NaCl). The washed cells were dispersed in 5 ml. of the same buffered saline.

Nucleic Acid (Treated or Untreated) Solution—The nucleic acid solution was prepared by mixing the nucleic acid dissolved in 0.15 ml. of buffered saline (as described above), 0.15 ml. of 2 per cent KH_2PO_4 (pH 7.0), 0.3 ml. of 0.2 per cent $MgSO_4$, and 0.15 ml. of maltose (10 mg./ml.) This solution was kept at 0°.

Titration for Hemolytic Activity—0.75 ml. of cell suspension added to 0.75 ml. of nucleic acid solution was mixed and divided into three small test tubes. During this operation solutions were kept at 0°. After incubation at 37° respectively for 5, 15, and 30 minutes, each tube was taken out, chilled immediately at 0°, and kept at that temperatuer until the next day when it was titrated for hemolytic activity.

For the titration, the cells were removed from the above mixture by centrifugation, and the supernatant was diluted two-fold serially (1 :2, 1 :4, 1 :8, 1 :16, *etc.*). To each dilution of nucleic acid mixture an equal volume of 3 per cent rabbit red cell suspension was added and then it was incubated at 37° for 2 hours.

The hemolytic units were expressed as the multiple dilution value which produced 50 per cent hemolysis. In almost all experiments of measuring the potencies of hemolysin formation of RNA, the concentration of RNA in the starting sample was so adjusted that the measured hemolytic units may lie between 50 and 500.

The hemolytic units were finally expressed in terms of dilution of a solution whose concentration was 1 mg./ml. In this way, the hemolysin-forming potencies of various nucleic acids could be compared with one another.

II. The Expression of the Degree of Degradation

It is desirable to express the degree of degradation as precisely as possible. For this purpose, many criteria for estimation of degree of hydrolysis have been reported (*9–11*).

Because of very wide range of degradation studied by us, the proportion of secondary phosphoryl dissociations was determined by titration of a 0.4 per cent solution of the ribonucleate in 0.10 M NaCl with 0.01 N NaOH. The titer between pH 6.0 and 8.0 was doubled and considered to represent the maximal amount of secondary phosphoryl groups (*11*).

The total phosphorous content was determined by Allen's method (*12*). The degree of degradation was indicated by the ratio "Total P/Secondary dissociable P."

III. *Alkaline Hydrolysis of the Nucleic Acid*

All of the following operations were carried out in a cold room (4°). To the previously ice-cooled 12.5 per cent solution (pH 7.0) of nucleic acid (prepared by Clark Schryver's method (*13*)) 1/4 its volume of ice cold 5 *N* NaOH was added with stirring. After a given time, the solution was neutralized with an equivalent volume of cold 5 *N* HCl with vigorous stirring, while the temperature was kept at 0∼4°. The solution was adjusted to pH 4.5. After 5 minutes an equal volume of cold acetone was added with stirring at 0°. Further 15 minutes later, the precipitate was collected by centrifugation, washed with cold acetone twice, and then dried in a vacuum desiccator. Table I shows the potencies of these samples ; the third line indicates the degradation degree.

As shown in Table I, the hemolysin-forming potency was enhanced gradually in the initial stage of hydrolysis under these above-mentioned conditions. At this stage, degradation is still barely measurable.

TABLE I

Hemolysin Forming Potencies and Degradation Degrees of Alkali-Hydrolyzed Ribonucleic Acids

Yeast RNA was hydrolyzed with 1.0 *N* NaOH for various periods at 0°. The degradation degree was expressed in the term of "*Total P / Secondary dissociable P.*"

Time of hydrolysis (hours.)	0	1/6	1/3	1/2	1	3/2	3	6	24
Potency	80	100	100	250	300	280	280	270	8
Degree of degradation	6.5	6.8	7.0	6.8	6.1	5.5	3.9	3.3	1.9
Yield (%)	105	93	93	99	92	90	90	90	70

Thus it is shown that any structural change of nucleic acid which increases its hemolysin-forming power occurs in initial stages. Loss of potency after more complete hydrolysis was also confirmed.

IV. Acetone Fractionation of the Untreated Nucleic Acid and of Slightly Hydrolyzed Samples

For obtaining further information concerning the nature of the hemolysin-inductive structure, the untreated nucleic acid (starting material of the alkaline hydrolysis) and the 30-minutes alkali-hydrolyzed nucleic acid were fractionated with acetone.

The nucleic acid solution was hydrolyzed with 1 *N* NaOH for 30 minutes at 0°, and neutralyzed to pH 4.5 (final content of RNA 8.3 per cent), then cold acetone was added up to 10 per cent concentration, and thereafter the precipitate was collected by centrifugation and treated as described in *Section III.* Successive acetone fractionation were carried out for the supernatant with the concentrations 10-20 per cent, 20–30 per cent, 30–50 per cent, and 50–75 per cent, and finally the same procedure was repeated with 10–20 per cent and 20–30 per cent fractions. In any case the fractionations were carried out at the temperature kept at 0∼4°. Untreated nucleic acid was fractionated in 1 *M* NaCl in a

TABLE II

Hemolysin Forming Potencies and Degradation Degrees of Acetone Fractionated Untreated and Alkali-Hydrolyzed RNA

Acetone concentration		0–10%	10–20%	20–30%	30–50%	50–75%
Potency	untreated RNA	——*	380**	230**	40	0
	treated RNA	400	700	300	120	20
Degradation degree	untreated RNA	——*	9.4**	9.6**	7.9	3.2
	treated RNA	5.0	6.1	6.2	4.9	3.2

* So small amount of this fraction was obtained that we could not examine for this fraction.

** After second fractionation of 0–30 per cent fraction, as we could not not obtain clear cut fractions in the first time.

Second fractionation of alkali-treated RNA.

Acetone concentration		0–10%	10–20%	20–30%	30–50%
Potency	10–20% fraction of first fractionation	650	500	300	
	20–30% fraction of first fractionation.		420	270	70

similar way. The hemolysin-forming potencies and degradation degrees are given in Table II.

In both experiments, it seems clear that only rather large molecules have hemolysin-forming potency.

These results will be considered together with those of enzymatic hydrolysis which will be presented in the following paper.

SUMMARY

1. The hemolysin-forming potencies of the differently alkali-hydrolyzed nucleic acids were studied.

2. In the early stages of alkaline hydrolysis slight enhancement of the potency was observed.

3. Through acetone fractionation of unhydrolyzed nucleic acid and of that briefly treated with alklai, the fractions were tested for hemolysin-forming potency. In both cases, it appears that only rather large molecules have such potency.

The authors wish to thank Prof. F. Egami for his helpful suggestions in this work. It is a pleasure to acknowledge several helpful discussions with Dr. Y. Yagi.

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The Journal of Biochemistry Vol. 43, No. 6, 1956

BIOCHEMICAL STUDIES ON STREPTOLYSIN S.

II. EFFECTS OF RIBONUCLEASE DIGESTION OF RIBONUCLEIC ACID ON THE HEMOLYSIN FORMATION

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(Received for publication, August 6, 1956)

It was found by Bernheimer and Rodbart that the hemolysin-forming activity of yeast ribounucleic acid was greatly enhanced by ribonuclease digestion (*1*). The finding was confirmed and extrended by Hosoya, Egami and their coworkers (*2–3*). However, the mechanism of the enhancement remained unclarified. It is the purpose of the present investigation to make more systematic studies on the problem, taking into consideration recent informations on the specificity of ribonuclease.

EXPERIMENT AND RESULTS

I. Determination of Hemolysin-Forming Potency

The method employed and the way of expressing the results were essentially the same as described in the preceding paper (*4*). In the preliminary experiments on the titration for hemolytic activity, it was found that to obtain maximum hemolysin formation the ribonuclease hydrolyzed ribonucleic acid (RNA) requires longer incubation of RNA solution with cells than the alkali-hydrolyzed RNA. This finding appears significant, though not easily explicable at present. In this experiment, incubation time to determine the hemolysin-forming potency of the RNase-hydrolyzed RNA was fixed at 30, 90, and 150 minutes.

II. Hydrolysis of the Untreated RNA for Various Periods

To the solution of 13 g. of commercial yeast RNA in 260 ml. (pH 7.4), 13 mg. of RNase (McDonald's B fraction (*5*)) was added and the resulting solution was incubated at 37°. During the course of digestion, the pH of the reaction mixture was frequently adjusted to

7.2–7.4. At certain intervals 40 ml. each of the reaction mixture was taken out, and immediately adjusted to pH 4.4 and then cooled down to 0°. After the addition of NaCl so as to get a concentration of 1 *M.*, cold acetone was added to the mixture until 20 per cent concentration was obtained. Letting the reaction mixture stand for 15 minutes at 0°, the resulting precipitate was collected by centrifugation, washed with cold acetone twice and dried in a vacuum desiccator. Successive fractionation was carried out for the supernatant with acetone concentrations of 20–30, 30–50, and 50–75 per cent.

Only in the case of 18-hour hydrolysis, 110 ml. of reaction mixture was dialyzed in a cellophane bag against 1 *l.* of distilled water at 4° overnight. The inner liquid (175 ml.) was adjusted to pH 4.4 and fractionated with acetone as described above. The outer liquid of the dialysis was concentrated to 60 ml. in vacuum at the temperature below 45° and fractionated with acetone in a similar manner.

The hemolysin-forming potencies of these samples are shown in Table I.

TABLE I

Hemolysin Forming Potencies after Ribonuclease Hydrolysis

Yeast RNA was hydrolyzed by 1/1000 of its weight of RNase, then fractionated with cold acetone.

Period of hydrolysis (*hours*)	0	1	2	4	8	18*	18**
Acetone conc.							
0–20%	380	6000	9000	7000	6000	14000	8
20–30%	230	16000	16000	16000	10000	28000	10
30–50%	40	8000	12000	10000	10000	21000	4
50–75%	0	40	120	80	40	0	0
Potency × material yield/1000,000/2g. RNA	2.1	120	134	105	103	130	

* Fraction obtained from inner liquid.

** Fraction obtained from outer liquid.

Each of these samples had remarkably high potency, and within the period of hydrolysis the dependency of potency on the degradation time was not clearly recognized. The nondialysable residue of 18-hour hydrolysis had the highest potency, while the dialysable material had only little potency.

III. RNase Hydrolysis of Alkali-Prehydrolyzed RNA

In order to confirm the effect of RNase on the potency of RNA, alkali-hydrolyzed RNA was hydrolyzed again with RNase.

RNA precipitated at 0–50 per cent acetone concentration after 60-minute hydrolysis with 1 *N* NaOH at 0° was chosen as a substrate.

To the solution of 2 g. of above-described RNA in 40 ml. (pH 7.4), 2 mg. of RNase was added. The temperature of incubation was 37°. After 4-hours' incubation acetone fractionations were carried out as described in *Section II.* Results are given in Table II.

TABLE II

Hemolysin Forming Potencies of Ribonuclease Hydrolyzed Alkali-Prehydrolyzed RNA

Yeast RNA which was previously hydrolyzed with 1 *N* NaOH for 1 hours at 0° was hydrolyzed with 1/1000 of its weight of RNase for 4 hours, then fractionted with cold acetone.

	Acetone fractionations after RNase hydrolysis				
	Substrate	0–20%	20–30%	30–50%	50–75%
Potency	56*	0	4000	3000	0
Potency × material yield /100,000	1.1	0	16	13.5	0

* Commercial yeast RNA used for alkali-prehydrolysis was not of the same lot as for other alkali-hydrolysis experiments.

It can be seen from these data that even in the case of alkali-prehydrolyzed RNA RNase hydrolysis greatly enhanced its hemolysin forming potency, while in the case of RNA pretreated with alkali drastically (hydrolyzed in the same condition for 24 hours), enhancement of the potency was not observed by subsequent RNase hydrolysis.

IV. RNase Hydrolysis of Acetone-Fractionated Alkali-Prehydrolyzed RNA

For further confirmation concerning the nature of RNAase-treated RNA, RNase hydrolysis was carried out for RNA which was acetone fractionated after 30-minutes' prehydrolysis with 1 *N* NaOH at 0°. (Details of alkali-hydrolysis was given in the previous paper.)

In the case of the fraction at 20–30 per cent acetone concentration after preliminary alkali-hydrolysis, 2 mg. of RNase was added to the solution of 2 g. of the sample 40 ml. in volume (pH 7.2). This was incubated at 37° and after 4.75 hours fractionated again with acetone as described in *Section II.*

In the case of 30–50 per cent fraction, 800 mg. of the sample was dissolved in 10 ml. of water. pH was adjusted to 8.0 and, after addition of 1 mg. of RNase, it was incubated at 37°. After 1, 2, and 4 hours alkali consumption were determined by the use of *N*/10 NaOH (Fig. 1).

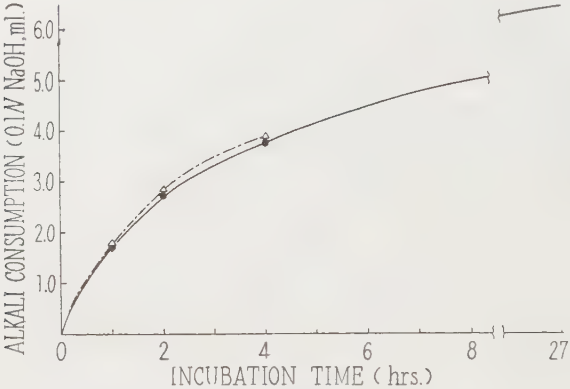

FIG. 1. RNase hydrolysis of 30–50 per cent fraction after alkali-prehydrolysis.

800 mg. of sample was dissolved in 25 ml. of water (pH 8.0) hydrolyzed with 1 mg. of RNase at 37°. △—·—△ 4 hours-hydrolysis. ●—●—● 27 hours-hydrolysis.

After 4-hours' incubation pH of the reaction mixture was adjusted to 7.0 and 1/2 volume of the reaction mixture was dialyzed overnight against the distilled water in a cellophane bag at 4°, the other half of the mixture being lyophilized.

Under the same condition 27-hour RNase-hydrolysis was also carried out.

In the case of 50–75 per cent fraction, 500 mg. of the sample was dissolved in 10 ml. of water, then hydrolyzed with 1.5 mg. of RNase in a similar way (Fig. 2). After 4-hours' hydrolysis pH was adjusted to 7.0 and the reaction mixture was lyophilized.

Table III shows the potencies of these samples.

As shown in Table III, RNase hydrolysis also enhanced the hemoly-

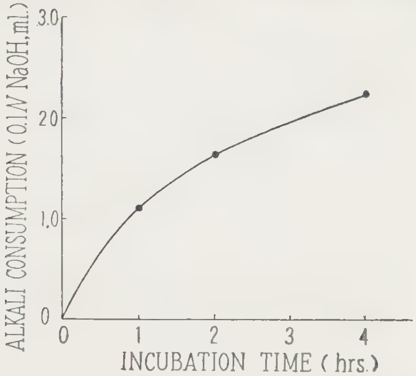

FIG. 2. RNase hydrolysis of 50–75 per cent fraction after alkali-prehydrolysis.

500 mg. of sample was dissolved in 10 ml. of water (pH 8.0) and hydrolyzed with 1.5 mg. of RNase at 37°.

TABLE III

Effects of Ribonuclease Hydrolysis on the Hemolysin Forming Potencies of Fractionated RNA after Alkali-Prehydrolysis

a) RNase hydrolysis of 20–30 per cent fraction of the first acetone-fractionation after alkali-prehydrolysis.

	Starting material	Acetone fraction after 4-hour RNase hydrolysis			
	300	0–20%	20–30%	30–50%	50–75%
Potency	300	8000	12000	12000	80
Potency × material yield /100,000.	6	29.6	44.4	32.4	

b) RNase hydrolysis of 30–50 per cent, and 50–75 per cent fractions of the first aceton-fractionated RNA after alkali-prehydrolysis.

	Potency after RNase hydaolysis			
Periods of hydrolysis (hrs.)	0	4	4*	27
30–50% fraction.	120	5000	5500	5500
50–75% fraction.	20	1000		

* Nondialysable material after 24-hours' dialysis.

sin-forming potency remarkably.

V. The Effect of Diluted Acid on the RNase-Hydrolyzed RNA

It has been indicated that the treatment with 0.1 *N* HCl for 4 hours at 20–25° destroys the cyclic phosphate bond in RNA molecules which formed during the course of hydrolysis with RNase (*6*). Some of the samples derived by means of RNase hydrolysis was subjected to this treatment. In the case of the samples fractionated at low acetone concentration, precipitation was found to occur in such a low pH medium. It may be impossible to remove the effect of this precipitation on the rate of the reaction, although the reaction mixture was vigorously stirred. Reduction of the hemolysin-forming potency to about one half of their starting potency was observed in all cases excepting the case of the alkali-prehydrolyzed RNase-treated 50–75 per cent fraction (described in *Section IV*).

VI. Determination of the Degree of Degradation

For some of the samples chosen from the foregoing experiment, the degree of degradation was measured (the method and the definition were the same as described in the preceding paper (*4*)).

Table IV shows the results.

These results show that degradation of RNA by RNase hydrolysis was fairly rapid.

Through comparison of these results with those of alkaline hydrolysis of RNA (*4*), it was found notable that the RNase-hydrolyzed RNA has remarkably higher potency than the alkali-hydrolyzed RNA having nearly the same degradation degree. For example, as shown in the preceding paper (*4*), after 6-hour hydrolysis with 1 *N* NaOH at 0°, the degree of degradation came out to be 3.3 and the potency 270, while with the same degree of degradation, the RNase-hydrolyzed RNA sample showed the potency 28000.

It is clear, therefore, that the enhancement of the hemolysin-forming potency of RNA is not only due to the depolymerization, but to the specific structure caused by the RNase hydrolysis. This information was also confirmed by the studies on RNase hydrolysis of alkali-prehydrolyzed RNA.

TABLE IV

The Degradation Degrees of the Ribonuclease Hydrolyzed RNA

Sample.	"Total P / Secondary dissociable P."
(*Section II.*)	
Acetone 20–30% fraction after RNase hydrolysis.	
Period of hydrolysis.	
0 hr.	9.6
2 hrs.	3.5
18 hrs. (dialysable)	2.5
18 hrs. (nondialysable)	3.3
(*Section III.*)	
Acetone 30–50% fraction of alkali-prehydrolysis after RNase hydrolysis.	
Period of RNase hydrolysis.	
0 hr.	4.9
4 hrs.	2.6
27 hrs.	1.9
Acetone 50–75% fraction of alkali-prehydrolysis after RNase hydrolysis.	
Period of RNase hydrolysis.	
0 hr.	3.1
4 hrs.	1.9
(RNA lyophilized after 94-hours' RNase digestion accompanied with dialysis.	3.3)

SUMMARY

1. The hemolysin-forming potency of the RNase-hydrolyzed RNA was studied in some detail.

2. The enhancement of the potency by the RNase hydrolysis was confirmed.

3. The hemolysin-forming potency of alkali-hydrolyzed RNA was also enhanced by the RNase hydrolysis.

4. The hemolysin-forming potency of the slightly alkali-prehydrolyzed fraction which is almost inactive and is composed of smaller molecules was also enhanced by RNase hydrolysis.

5. Comparison between the potencies of alkali-hydrolyzed RNA and of RNase-hydrolyzed RNA having nearly the same degradation

degree, showed that the remarkable enhancement of hemolysin-forming potency by RNase hydrolysis is closely connected with the specificity of the RNase.

The authors wish to thank Prof. F. Egami for his encouraging interest and advices during the course of this work.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

II. EFFECT OF AMINO ACID ANALOGUES ON AMYLASE FORMATION BY BACILLUS SUBTILIS; AN APPARENT COMPETITION BETWEEN AMYLASE FORMATION AND NORMAL CELLULAR PROTEIN SYNTHESIS

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In order to clarify the mechanism of protein biosynthesis the formation of amylase by *B. subtilis* has been selected as an experimental system (*1*, *2*). In a previous paper it was shown that the phase of active amylase formation by growing cells of this bacterium is distinct from that of cell multiplication and takes place only in the old cells. The concept of abnormal biosynthesis of amylase was proposed (*1*) from this and other experimental results especially because of the apparent relationship between amylase production and anaerobic lysis (*3*, *4*). Studies have been undertaken from this point of view along with studies using a lysozyme lysed-cell system (*2*). In this paper an apparent competition between amylase formation and normal cellular protein synthesis will be reported, together with the peculiar behavior of amino acid analogues in this system.

METHODS

B. subtilis H, wich has been used throughout the previous studies, was also used in this investigation. Cells were grown in Soy medium at 30° for 20 to 24 hours with shaking, (that is, until cell multiplication has already finished and amylase has begun to appear in the medium), harvested, washed once with salt solution C and resuspended in the same salt solution. Using this washed cell suspension, the amylase formation was examined at 30° under various experimental conditions. Usually the total volume of the reaction mixture was made twice the volume of the washed cell suspension used. The cell weight used in the reaction was about 6 to 12 mg. per ml. of reaction mixture.)

The composition of Soy medium is as follows: soluble starch 8 per cent., Na citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_4 \cdot 7H_2O$ 0.002 *M*, $CaCl_2$

0.001 *M*, ethyl alcohol 1 per cent (*v*/*v*), soybean extract (50 g. of soybean were extracted with about 250 ml. of 0.1 per cent NaOH by boiling for 1 hour. This extract was used per 1 litre of medium.), pH=7.2. Salt solution C: $Na_2HPO_4 \cdot 12H_2O$ 0.225 *M*, KCl 0.03 *M*, $MgSO_4 \cdot 7H_2O$ 0.003 *M*., $CaCl_2$ 0.0015 *M*., pH=7.2.

Amylase was assayed by the method of Hagihara (*5*). This method allows the determination within an error of about ±5 per cent. Radioactivity was determined by the conventional Geiger-Müller counter with an end window. The counting data were corrected for self absorption.

RESULTS

Acceleration of Amylase Production by Boiled Cell Extract and Inhibition of This Effect by A Glucose-Amino Acid Mixture

The effect of several substances on amylase production was examined using washed cell suspensions. The washed cell suspension alone produces an appreciable amount of amylase without any further additions. This production cannot be considered as a mere liberation of preformed amylase within the cell for the following reasons: (1) little amylase can be found within the cell when lysed with lysozyme, (2) several metabolic inhibitors, *e.g.*, cyanide (0.0017 *M*), chloramphenicol (10 μg./ml.), streptomycin (100 μg./ml.) and toluene inhibited this process almost completely. The addition of a glucose (0.5 to 5 per cent) and amino acid mixture (casein trypsin hydrolysate 5 mg./ml. or casein acid hydrolysate fortified with tryptophan, cysteine and methionine 4 mg./ml ; the effect is the same in both instances.) had no effect during the initial period of 3 to 4 hours (*cf.* Fig. 1). Even a distinct inhibition was sometimes observed in this period, though the cell material became much more than that of the control flask. The addition of amino acid analogues (for example, 0.01 *M* ethionine) was not only unable to inhibit amylase formation but sometimes showed a considerable activating effect when added to the flask containing the glucose and amino acid mixture (Fig. 1). Among the many substances tested, the most effective for amylase formation was a boiled cell extract (100°, 10 to 20 minutes) of cells cultivated in the phase of active amylase production. This extract contains a factor necessary for the formation of amylase in the lysozyme lysed-cell preparation as reported previously (*2*). It is a non-dialysable substance(s), precipitated by three volumes of ethanol and also by trichloroacetic or perchloric acid (final concentration 0.3 *N*). A preliminary fraction diagram of this factor is presented in Fig. 2. The factor at the stage B_{sp} was used in most of the experiments in this paper.

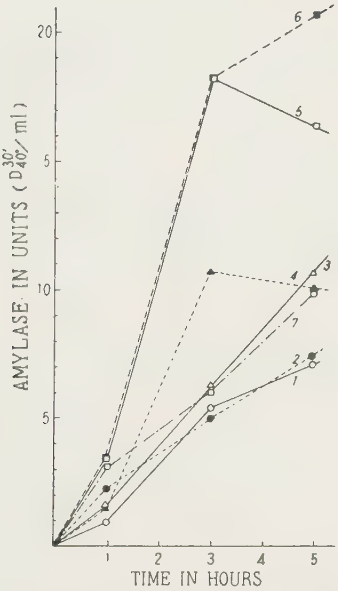

FIG. 1. Amylase formation by washed cell suspension of *B. subtilis*: effect of factor B_{sp}, amino acid analogues and glucose and amino acid mixture.

Curve 1: no addition.
Curve 2: ethionine (0.01 *M*) plus 5-methyltryptophane (0.005 *M*)
Curve 3: glucose (1 per cent) plus casein tryspin hydrolysate (4 mg./ml.).
Curve 4: same as 3, and ethionine (0.01 *M*) plus 5-methyltryptophane (0.005 *M*).
Curve 5: factor B_{sp} (its nucleic acid content was 0.4 mg./ml.).
Curve 6: same as 5, and ethionine (0.01 *M*) plus 5-methyltryptophane (0.005 *M*).
Curve 7: same as 5, and glucose (1 per cent) plus casein trypsin hydrolysate (4 mg./ml.).

In Fig. 1 the activating effect of this factor on amylase formation is presented. Amylase formation in the presence of this factor was not inhibited by amino acid analogues (ethionine 0.01 *M* plus 5-methyltryptophan 0.005 *M*), but was greatly inhibited by the addition of glucose (1 per cent) plus casein trypsin hydrolysate (4 mg./ml.) (Fig.

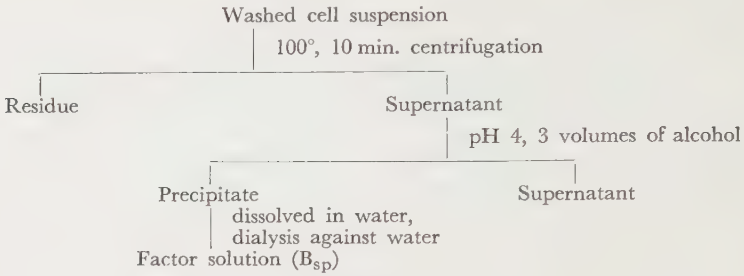

FIG. 2. Fractionation diagram of the factor.

1). This inhibition was slight when the amino acid mixture was used alone and considerable with glucose alone, but the most severe inhibition was observed when amino acid mixture and glucose were added together (results obtained in another experiment). From these results it was supposed that amylase synthesis is antagonized by the synthesis of normal cellular protein, the inhibition by the glucose and amino acid mixture being due to normal cellular protein synthesis. To test this postulate, amino acid analogues were used to counteract the synthetic reactions due to the glucose and amino acid mixture. The experimental results presented in Table I clearly demonstrated that the analogues, in fact, could greatly suppress the inhibitory action of the glucose and amino acid mixture.

Effect of Ethionine on the Incorporation of S^{35}-Methionine into Cellular Protein and on the Formation of Amylase

As shown in the previous section, amylase formation was not inhibited by the amino acid analogues tested, *i. e.*, by ethionine (0.03 *M*), norleucine (0.015 *M*) and 5-methyltryptophan (0.005 *M*). Both ethionine and 5-methyltryptophan are well-known amino acid analogues. Norleucine (0.45 mg./ml.) was shown to be an effective antagonist of methionine in *Bacillus mycoides* by Foster *et al.* (*6*, *7*). The effect of ethionine and norleucine on growth of *B. subtilis* H was then examined. Ehionine partially inhibited growth at a concentration of 0.003 *M* or above, but no inhibition was observed with norleucine at a concentration of 0.8 mg./ml. Though the above results seem to indicate the non-involvement of amino acid in amylase formation in our system, further examination was performed using S^{35}-labeled methionine.

S^{35}-Methionine was added to the washed cell suspensions together

TABLE I

Inhibition of Amylase Formation in the Presence of Factor B_{sp} by Glucose and Amino Acid Mixture and Its Suppression by Amino Acid Analogues

Additions \ Flask no.	1	2	3	4	5	6
Factor B_{sp}*	−	−	−	+	+	+
Glucose (0.5%) plus amino acid mixture**	−	+	+	−	+	+
Ethionine (0.01 *M*) plus norleucine (1 mg./ml.)	−	−	+	−	−	+
Amylase formed ($D^{30'}_{405}$/ml.)	6.9	5.4	5.6	12.7	4.5	9.2
% of inhibition				−	65^{+}	−
% suppression of inhibition				−	−	57^{++}

* Its nucleic acid content is 0.34 mg./ml. in the reaction mixture.

** Casein acid hydrolysate (4 mg./ml.) fortified with DL-tryptophan (0.4 mg./ml.), DL-methionine (0.4 mg./ml.) and L-cysteine hydrochloride (0.25 mg./ml.).

The reaction was performed for 6 hours at 30°.

$$^{+}\ \frac{12.7-4.5}{12.7}\times 100=65$$

$$^{++}\ \frac{9.2-4.5}{12.7-4.5}\times 100=57$$

with various other additions. At intervals samples were withdrawn and both the amount of amylase and S^{35}-methionine taken up in the protein fraction were determined. Thus the effect of ethionine on these two processes were examined simultaneously. The results are presented in Table II. Ethionine greatly inhibited (40 to 60 per cent) the incorporation of S^{35}-methionine, yet no inhibition of amylase formation was observed within experimental error of 10 per cent (even the activating effect was observed in the early phase).

DISCUSSION

Following observations lead us to consider amylase formation by this bacterium as a special physiological process, a kind of abnormal biosynthesis, distinct from and competing with normal cellular protein synthesis.

TABLE II

Effect of Ethionine on the Incorporation of S^{35}-Methionine into the Protein Fraction and on the Formation of Amylase

Time (*hrs*)	Additions*	Ethionine (*M*)	Incorporation of S^{35}-methionine		Formation of amylase	
			Total count (*c.p.m.*)	Inhibition (*%*)	Amount $(D^{30'}_{40^\circ}/ml.)$	Inhibition(+) (or activation (−)) (%)
2	H	—			4.3	—
		0.01			5.4	−26
		0.03			5.4	−26
	G	—	6,100	—	6.0	—
		0.01	3,600	40	7.3	−22
		0.03	3,100	50	6.6	−10
	B	—	12,100	—	5.4	—
		0.01	5,100	58	6.0	−11
4	H	—			9.0	—
		0.01			8.1	+10
		0.03			9.0	0
	G	—	26,100	—	9.0	—
		0.01	15,000	43	9.0	0
		0.03	11,800	55	8.1	+10
	B	—	46,300	—	16.7	—
		0.01	22,800	51	16.7	0
6	H	—			11.0	—
		0.01			11.0	0
		0.03			10.0	+9
	G	—	41,200	—	15.0	—
		0.01	25,000	40	15.0	0
		0.03	17,700	57	13.5	+10
	B	—	54,600	—	43.0	—
		0.01	29,700	46	39.0	+9

* Composition of the reaction mixture: washed cell suspension 5 ml., ethionine as indicated in the table and the following additions: H; no addition; G; glucose (1 per cent) plus casein trypsin hydrolysate (4 mg./ml.); B; factor B_{sp} (nucleic acid content 270 μg./ml.). Besides, S^{35}-DL-mithionine (200 μg., 3.75×10^5 c.p.m.) was added to each flask of G and B. Total volume 10 ml. pH=7.2.

The reaction was performed at 30° under aeration. At the 2nd and

(a) A close relationship exists between amylase formation and " anaerobic lysis " described in a previous paper (*3*).

(b) The phase of active amylase formation by grwoing cells of this bacterium is distinct from that of cell multiplication and immediately followed by the phase of autolysis as already reported (*1*).

(c) The glucose and amino acid mixtures, while they promote normal cellular protein synthesis, inhibit amylase formation, especially its formation in the presence of an active factor extracted from the cells.

(d) Amino acid analogues do not inhibit amylase formation though they inhibit the incorporation of amino acid into the cellular protein. When amylase formation is inhibited by normal cellular protein synthesis, analogues suppress this inhibition by inhibiting cellular protein synthesis.

As to the active factor obtained from the boiled cell extract, studies are now in progress using a lysozyme lysed-cell preparation system and will be reported later (*8*). This factor is a heat stable, non-dialysable factor and is found in the nucleoprotein fraction. Using S^{35}-labeled cells, and an S^{35}-labeled factor preparation, it was clearly demonstrated that this factor is not a precursor of amylase protein, at least with respect to the sulfur of the amylase protein (*cf.* footnote of page 848), but rather it must be considered as an activator (*8*). The large amount of amylase produced in response to this active factor comes solely from the substances within the cell (or from the lysozyme lysed-cell preparation in the case of cell-free system). Exogenous amino acids do not seem to be the direct precursor.

From the fact that amino acid analogues can not inhibit amylase formation and yet greatly inhibit the incorporation of S^{35}-methionine into the cellular protein, the following two possibilities may be con-

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4th hours, aliquots were withdrawn and the protein fraction was first precipitated by the addition of an equal volume of 10 per cent trichloroacetic acid in the cold, freed of nucleic acid by two extractions of the latter with hot 5 per cent trichloroacetic acid at 100° for 5 minutes, washed with 80 per cent alcohol, dried and counted. At the 6th hours cells were separated from the medium, and the radioactivity of the protein fraction was determined according to the same method as described above.

sidered* : 1) Amylase formation involves amino acid incorporation, but this process is a special one insensitive to ordinary amino acid analogues. It competes with normal protein synthesis for amino acid. Thus the inhibition by glucose plus amino acid mixture of amylase formation and its reduction by amino acid analogues may be explained at the stage of amino acid utilisation. 2) Amylase formation does not involve free amino acid utilisation but rather utilizes preformed complex precursor substance(s). An active factor obtained from the boiled cell extract is concerned with the activation of this reaction, that is, some terminal stage of serial reactions leading from amino acids to active enzyme. The competition between amylase formation and normal cellular protein synthesis will take place at this stage. The possible role in this competition of an active determinative agent, such as a nucleic acid, seems to be very attractive hypothesis.

Of the above two possibilities the former may be considered as less likely, but the final decision will be made either by adequately designed isotopic experiments or by experiments with the cell-free system.

Recently, Rotman and Spiegelman (*9*), and Hogness *et al.* (*10*) have shown that the β-galactosidase in *E. coli* is not synthesized from any pre-existing compounds within the cell and furthermore, that protein synthesis in growing cells of *E. coli* is virtually irreversible. The formation of amylase in our system shows very different and peculiar aspects. It does not seem to involve the utilisation of exogenous amino acid, and in fact its direct formation from preformed complex precursor may be considered as a likely process. The difference may be understood from the fact that their system is concerned with adaptive enzyme synthesis by growing cells of a gram negative bacterium, *E. coli*, while our system is concerned with exoenzyme synthesis by a gram positive bacterium, *B. subtilis*. This exoenzyme synthesis is performed in a stationary phase, when normal cellular protein synthesis has already finished. Many special types of protein synthesis, including the lethal synthesis which causes the death of the bacterial cell, leading to the death of population as a whole, take place and transformation or disintegration and resynthesis of preformed complex substances into these special proteins may be performed in this period. Disintegration of cellular ptoein to form spore protein is an example of this kind of process. Exoenzyme formation in general, and exotoxin formation also, may be regarded as examples of such physiological processes.

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* Hokin (*11*) reported that methionine, which was stated to be the only " essential " amino acid not present in the α-amylase of pancreas, was not needed for amylase synthesis by pigeon-pancreas slices. However, crystalline α-amylase of *B. subtilis* H, obtained from the medium using $S^{35}O_4$ as the sole source of S, was clearly shown to contain S^{35}-methionine (*12*). Therefore, the experiments using S^{35}-methionine, or methionine analogues, ethionine and norleucine, presented in this paper are justified.

The insensitivity of the amylase forming system to amino acid analogues was also confirmed recently by Fukumoto *et al.* (*13*).

SUMMARY

Amylase formation by washed cell suspensions of *Bacillus subtilis* H was studied. A glucose and amino acid mixture, while it promoted normal cellular protein synthesis, showed no promoting effect on amylase formation during an initial period of 3 to 4 hours.

The addition of ethionine did not inhibit amylase formation, although it greatly inhibited the incorporation of S^{35}-methionine into cellular protein. Sometimes even a considerable activating effect was observed when ethionine was added alone or together with the glucose and amino acid mixture. Other analogues tested, 5-methyltryptophan and norleucine, also did not inhibit amylase formation.

Active factors, prepared from boiled cell extract, markedly promoted the formation of amylase. This process was greatly inhibited by the addition of the glucose and amino acid mixture. This inhibition was partially suppressed by the addition of amino acid analogues (ethionine plus norleucine).

The concept of competition between amylase formation as a kind of abnormal biosynthesis and normal cellular protein synthesis was proposed and discussed.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

ENZYMATIC PREPARATION OF OPTICALLY ACTIVE ESSENTIAL AMINO ACIDS

I. THE PREPARATION OF L-PHENYLALANINE

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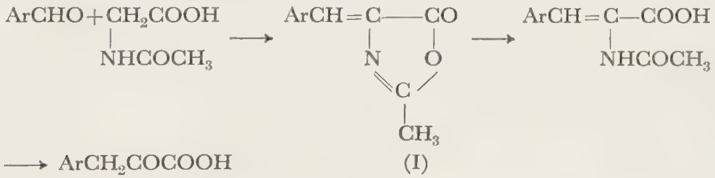
Preparation of the essential amino acids in natural form is very important for research works on nutrition. Recently it was confirmed that these amino acids can readily be prepared from the corresponding α-keto acids by transaminase. Although a number of these α-keto acids can be prepared by various methods, none is obtained in economical cost.

The keto analogues of aromatic amino acids, such as phenyl alanine (*1*) and tryptophan (*2*), have generally been prepared by hydrolysis of unsaturated azlactone derivatives as follows (I).



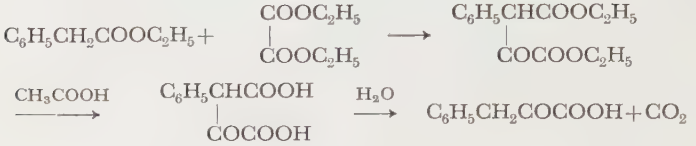
(I)

Although this method, especially in the case of phenylpyruvic acid, gives fairly good yield, its practical availability is decreased because of expensiveness of the starting material and of many steps of reactions.

Wislicenus (*3*) demonstrated that phenylpyruvic acid could be obtained by acid hydrolysis of ethyl phenyloxalacetate, the condensation product of ethyl phenylacetate with diethyl oxalate.

In author's experiment, however, it was confirmed that the yield was only 10–15 per cent in this method.

It was now found that when ethyl phenyloxalacetate was heated with acetic acid in the presence of a small amount of catalyst, such as *p*-toluenesulfonic acid, ethyl acetate formed by esteritication was driven continuously from the reaction mixture and ethyl phenyloxalacetate was hydrolyzed to phenyloxalacetic acid and the latter was converted to phenylpyruvic acid in a good yield when it was dumped into water (II).



$$C_6H_5CH_2COOC_2H_5 + \begin{array}{l}COOC_2H_5\\ |\\ COOC_2H_5\end{array} \longrightarrow \begin{array}{l}C_6H_5CHCOOC_2H_5\\ \quad |\\ \quad COCOOC_2H_5\end{array}$$

$$\xrightarrow{CH_3COOH} \begin{array}{l}C_6H_5CHCOOH\\ \quad |\\ \quad COCOOH\end{array} \xrightarrow{H_2O} C_6H_5CH_2COCOOH + CO_2$$

(II)

In this paper, procedures are described for the preparation of phenylpyruvic acid from phenylacetic ester, and L-phenylalanine from phenylpyruvic acid by means of heart muscle transaminase.

EXPERIMENTAL

Reactions of Various Esters with Acetic Acid—From the results in Table I the following facts were observed.

1) Methyl ester is more reactive than ethyl ester in the case of phenylacetate.

2) Ethyl oxalylsuccinate does not react at all, while ethyl succinate and ethyl oxalacetate react with a moderate velocity.

3) Ethyl oxalylindoleacetate is more reactive than ethyl phenyloxalacetate and oxalylindoleacetic acid is decarboxylated to indolepyruvic acid under this condition. In the case of phenyloxalacetic acid, however, only partial decarboxylation is observed.

Preliminary Test of Glutamic Acid - Phenylalanine Transamination—

Table I

Reactions of Various Esters with Acetic Acid

Ester		Acetic acid g	*p*-Toluene sulfonic acid g	Reaction time hrs.	Yield	Unreacted ester
	g.				%	%
Ethyl phenylacetate	5	24	0.5	8	56	28
Methyl phenylacetate	5	24	0.5	8	87	0
Diethyl succinate	5	24	0.5	7	97	0
Ethyl oxalylsuccinate	5	24	0.5	8	0	100
Ethyl phenyloxalacetate	5	24	0.5	12	82*	0
Ethyl indolyloxalacetate	5	36	0.35	6	50*	0

* Yield of α-keto acid.

Table II shows that L-aspartic acid is more suitable than L-glutamic acid as amino donor in the Glutamic acid—Phenylalanine transamination when a small amount of L-glutamic acid is added in the reaction mixture.

This result is well explained in the following Equations (III)–(V)).

$$\text{Glutamic acid} + \text{phenylpyruvic acid} \rightleftarrows \alpha\text{-ketoglutaric acid} + \text{phenylalanine} \qquad \text{(III)}$$

$$\alpha\text{-Ketoglutaric acid} + \text{aspartic acid} \rightarrow \text{glutamic acid} + \text{pyruvic acid} + CO_2 \qquad \text{(IV)}$$

$$\text{Asparatic acid} + \text{phenylpyruvic acid} \rightarrow \text{phenylalanine} + \text{pyruvic acid} + CO_2 \qquad \text{(V)}$$

TABLE II

Yield of L-Phenylalanine in Transamination

	I	II	III	IV	V
L-Aspartic acid *mM*	0	0.61	0.61	0.61	0
L-Glutamic acid *mM*	0	0	0·061	0.305	1.83
Reaction time	Yield (%)				
hrs.					
20	10	25	28	30	61
48	19	53	55	55	64
70	20	64	66	78	70

Vessel contained 0.61 mM of phenylpyruvic acid, 3 ml., of 0.1 *M* phosphate buffer (pH 7.4) and 1.65 g. of pig heart tissue. Total volume was adjusted to 10 ml. and kept at 37°.

Preparation of Phenylpyruvic Acid—242 g. of diethyl oxalate was added to the alcoholic solution of sodium ethoxide (36.4 g. of sodium was dissolved in 500 g. of absolute ethyl alcohol) with vigorous stirring. After 10 minutes, 246 g. of ethyl phenylacetate was added in a rapid stream and the reaction mixture was allowed to stand overnight. The sodium salt of ethyl phenyloxalacetate deposited was filtered with suction and the salt was washed twice with 100 ml. of ethyl alcohol (From this mother liquor 17 g. of diethyl oxalate and 27 g. of ethyl phenylacetate were recovered).

The salt was suspended in 600 ml. of ice water, covered with 150 ml. of ether, and dilute sulfuric acid (42 ml. of concentrated H_2SO_4

in 200 ml. of water) was added to the suspension with stirring. After separating the ether layer, the aqueous layer was once extracted with 200 ml. of ether. The combined ether solution was dried over anhydrous sodium sulfate overnight and ether was distilled off. The residual oil, mainly consisting of ethyl phenyloxalacetate, weighed 355 g.

A mixture of 66 g. of the crude ester, 2 g. of para-toluenesulfonic acid and 160 g. of acetic acid was heated in a flask fitted with a fractionating column at such a rate as to distil off only ethyl acetate formed. Heating was continued for 12 hours and acetic acid was removed under diminished pressure. The crystalline residual substance, consisting of phenyloxalacetic acid and phenylpyruvic acid, was suspended in 250 ml. of water, and allowed to stand at 37° for 36 hours.

After cooling, white crystals of phenylpyruvic acid were collected with suction, washed with water and dried. It weighed 32 g. and showed m. p. 145–152°. Another 1.2 g. with m. p. 152–155° was obtained from the mother liquor. Total yield was 33.2 g. (81 per cent based on the phenylacetic acid consumed).

By recrystallization from chloroform pure phenylpyruvic acid with m. p. 155–157° was obtained.

Analysis. Calcd. for $C_9H_8O_3$; C, 65.85; H, 4.88
Found; C, 65.99; H, 4.93

Preparation of Enzyme Solution—90 g. of fresh pig heart muscle was blended with 240 ml. of water for 3 minutes. After centrifuging followed by filtration, 225 ml. of the clear solution was obtained. 1 ml. of this solution contains 1.76 mg. of protein N and 4 ml. of this solution has the activity of forming 30 mg. of L-phenylalanine per 20 hours in the condition given in the column IV of Table II.

Preparation of L-*Phenylalanine*—5 g. of phenylpyruvic acid, 5 g. of L-aspartic acid and 1.8 g. of monosodium glutamate monohydrate were added to 200 ml. of water. 20 per cent aqueous ammonia solution was dropped into the mixture until the clear solution has been obtained (pH 7.6). To this mixture 20 ml. of 0.2 *M* phosphate buffer (pH 7.6) and 225 ml. of the enzyme solution were added and then the total volume was adjusted to 500 ml. with water. The reaction mixture was placed in a 550 ml.-Erlenmeyer flask at 38°. After 96 hours, the mixture was heated to 80° for 10 minutes and the insoluble matter was filtered off. The clear filtrate was evaporated in vacuo to about 200 ml. and then the pH was adjusted to 6.1 with dilute hydrochloric acid. After removing the insoluble matter, the clear solution was passed through a column of Permutit DR (2×50 cm., Under this treatment the resine removed non-aromatic substances), and then the solution was followed by 800 ml. of water. The effluent was concentrated in vacuo to dryness and the residue was washed with a small amount of methyl alcohol and dried.

It weighed 3.2 g. (64 per cent) with m. p. 255°–260°. After one recrystallization from 45 ml. of water, this product melted at 273–5° and weighed 2.1 g.

Analysis.	Calcd. for $C_9H_{11}NO_2$;	C, 65.49;	H, 6.67;	N, 8.48
	Found;	C, 65.54;	H, 6.79;	N, 8.53

Since this product had a specific rotation, $[\alpha]_D^{25°} = -33.35°$ (209 mg. in 12 ml. of water, 1 dm., 25°), it was confirmed that this product was L-phenylalanine.

The author's best thanks are due to Prof. S. Akabori for his continued interest and advice.

SUMMARY

1. Phenylpyruvic acid was prepared by decarboxylation of phenyloxalacetic acid obtained by heating ethyl phenyloxalacetate with acetic acid and *p*-toluenesulfonic acid.

2. L-Phenylalanine was obtained from phenylpyruvic acid by heart muscle transaminase in an yield of 64 per cent.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

SPECIFICITY OF CRYSTALLINE BACTERIAL PROTEINASE

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(Received for publication, August 16, 1956)

A crystalline bacterial proteinase (BPN') prepared from the culture medium of *Bac. subtilis N'* through a cation exchange resin chromatography (*1, 2*), are being used for the determination of the ratio of denatured proteins to the native ones (*1, 3–6*), based on the fact that many denatured proteins are rapidly digested by this proteinase until the digestion product is no longer precipitated by concentrated trichloroacetic acid solution, while many native proteins are scarcely digested. On the other hand, the denatured proteins are rather difficult to be digested to the same extent as above by other neutral proteinases, such as trypsin and chymotrypsin. Since the fact seems to be caused by the different specificity among the above proteinases, the investigation presented in this paper has been attempted in order to obtain some knowledge on the specificity of the bacterial proteinase.

Many synthetic polypeptides have been used for the determination of the specificity of proteinases. However, it has also become possible to use proteins as a substrate for this purpose by the recent development in the techniques for end group analysis and sequence studies in peptides and proteins. Thus, insulin (*7, 8*), horse globin (*9*), and keratin (*10*) have been used for the determination of specificity of some proteinases. Using those techniques, the action of a crystalline proteinase from *Bac. subtilis* (subtilisin (*11*)) on oxytocin and fraction B of insulin was also investigated by H. Tuppy (*12*), and it was found that the enzyme had a broad specificity. In the present investigation, an attempt has been made to determine the substrate specificity of BPN' through the analysis of amino(*N*)- and carboxyl(*C*)-terminal amino acid residues of peptides produced from oxidized lysozyme and other proteins by the action of the enzyme.

MATERIALS AND METHODS

Crystalline Proteinase from Bac. subtilis N′ (BPN′)—A crystalline bacterial proteinase preparation, "Nagarse," supplied by the Nagase Co. Ltd., which was prepared according to the method of Hagihara, Matsubara, Nakai, and Okunuki (*1*, *2*) from the culture medium of *Bac. subtilis N′* using a cation exchange resin, Duolite C-10, was used after careful recrystallization from hydrous acetone and lyophilization.

Substrates—Crystalline egg white lysozyme prepared by the method of Alderton *et al.* (*13*) was oxidized by the method of Sanger (*14*), except that the reaction time with performic acid was 20 to 25 minutes. Oxidized lysozyme was slightly yellowish brown.

Denatured bacterial α-amylase was prepared by treatment with 6 *M* urea at 70° of the cystalline α-amylase obtained according to the method of Hagihara (*1*), and used after dialysis against dilute ammonia water in order to prevent coagulation.

Enzymatic Hydrolysis of Substrate—About 1 g. of the oxidized lysozyme was dissolved in 30 ml. of *M*/20 ammonium acetate buffer solution of pH 8.5. Twenty ml. of the substrate solution was mixed with 2 ml. of the same buffer solution containing 0.5 mg. of BPN′ and allowed to react at 30°. The substrate protein in the above reaction mixture partially remained insoluble but dissolved after several hours by the enzymatic action. An aliquot (10 ml.) of the reaction mixture was pipetted into a flask after 15 minutes and 24 hours, boiled for 10 minutes at 100° in order to terminate the enzymatic action, and then lyophilized. As a control experiment, 10 ml. of the oxidized lysozyme solution was mixed with 1 ml. of the proteinase solution previously boiled and treated as above (substrate: enzyme≒1300:1 *w*/*w*).

Qualitative and Quantitative Determination of the Terminal Amino Acid Residues

(*a*) *N-Terminal Residues*—The method of Sanger (*15*) was applied to identify the *N*-terminal residues of both undigested proteins and digestion products. Twenty-five mg. of each sample was dissolved in 5 ml. of 0.8 *M* sodium bicarbonate buffer solution of pH 8.5, mixed with fluorodinitrobenzene (FDNB) (0.05 ml. for control, 0.1 ml. for 15 minutes' digestion, and 0.3 ml. for 24 hours' digestion) dissolved in 10 ml. of ethanol, and the resulting mixture was shaken for 3 hours at room temperature. After removing ethanol *in vacuo*, 10 ml. of water was added and excess FDNB was extracted with ether. Ether was removed from the aqueous layer *in vacuo* and then the solution was neutralized with a few drops of conc. HCl. After addition of equal volume of conc. HCl to the solution, the mixture was hydrolyzed under refluxing for 12 hours at 115° to 120° (103° to 107° in the reaction flask). Dinitrophenyl (DNP)-amino acids were extracted with ether and dinitrophenol (DNP) was nearly removed by the method of Mills (*16*).

(*b*) *C-Terminal Residues*—The hydrazinolysis-DNP method of Akabori, and Ohno *et al.* (*17–20*) was applied. Since α,α-di-DNP-acidic amono acid mono (α, β, or γ)-hydrazide together with DNP-amino acids were extracted with sodium bicarbonate solution in their method (*19–21*), the hydrazide was converted to simple DNP-amino acid after the extract was hydrolyzed with 6 *N* HCl for 4 to 5 hours at 105°. The ordinary considerable changes of acidic amino acid residues by enzymatic action, hydrazinolysis, dinitrophenylation, and acid hydrolysis are shown in Fig. 1 (cf. Reference *21*). When the *C*-sides of Glu* and Asp in the protein, (a), are enzymatically hydrolyzed, the amount of DNP-Glu and -Asp should increase in the extract without treatment by acid. When the *C*-sides or amide bonds of Glu(NH_2) and Asp(HN_2), (c) or (d), are enzymatically hydrolyzed, the former should be converted to β- and γ-hydrazide derivatives and the latter to α-hydrazide derivatives, and thus the amount of DNP-Glu and -Asp should increase by treatment with acid. However, when the *C*-sides and amide bonds of Glu(NH_2) and Asp(NH_2), (c) and (d), are hydrolyzed at the same time, it would be difficult to detect whether the *C*-sides of Glu and Asp were enzymatically hydrolyzed or both *C*-sides and amide bonds of Glu(NH_2) and Asp(NH_2) were hydrolyzed. Accordingly the extract was divided into equal parts and one was chromatographed before and another after acid hydrolysis.

(*c*) *Chromatographic Separation and Quantitative Determination*—DNP-amino acids were mainly separated by paper chromatography according to the method of Levy *et al.* (*22*, *23*) and sometimes by chromatography on silica gel (*19*) or ion exchange resins (*24*), Amberlite IR-112 and IRC-50. The amount of separated DNP-amino acids was determined spectrophotometrically. DNP-Asp and -Leu were determined in a state of mixture with DNP-Glu and -ILeu, respectively, and DNP-Pro was not investigated due to experimental difficulty. In the case of *C*-terminal, quantitative determination was usually carried out after acid hydrolysis in order to prevent contamination of unknown hydrazide derivatives.

RESULTS AND DISCUSSION

1. N-Terminal Residues in Digestion Product from Oxidized Lysozyme—*N*-Terminal residues of peptides produced from oxidized lysozyme by the action of BPN′ are shown in Table I. According to Schroeder and Green (*25*), and Thompson (*26*), the *N*-terminal residue of lysozyme is 1 mole of Lys per protein molecule. In the present work the amount of Lys detected as *N*-terminal residue of oxidized lysozyme was very low, as shown in the third column in Table I. This low recovery of DNP-Lys might be attributed to experimental defect in the present work. Other amino acids, *i.e.*, Ser and acidic amino acids were found in a minute amount in the control sample. On comparing the 15-minutes' and 24-hours' digestions, it appeared that the ratio of each *N*-terminal residue was not changed so much during the course

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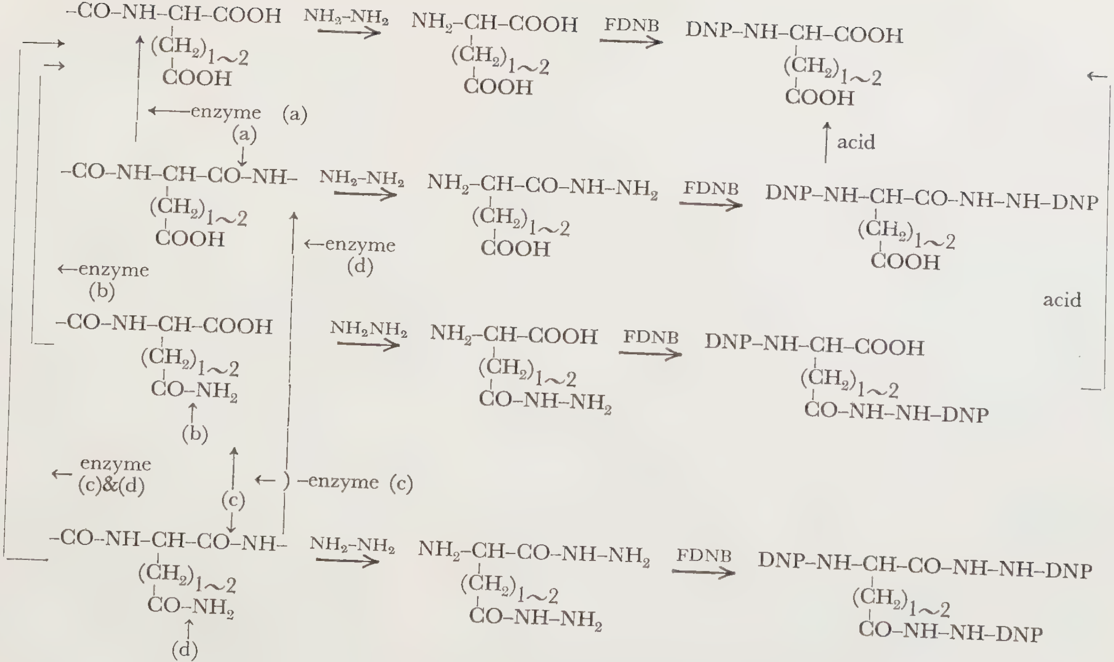
* The abbreviations for the amino acid residues suggested by Brand and Edsall (*33*) are used. $CySO_3H$ is the symbol for cysteic acid.



(into ethyl acetate phase)

FIG. 1. Changes of acidic amino acid residues by enzymatic action, hydrazinolysis, dinitrophenylation, and acidic hydrolysis. (a), (b), (c), and (d) are the bonds attacked by enzyme.

TABLE I

N-Terminal Residues in Digestion Product from Oxidized Lysozyme by the Action of BPN′

DNP-amino acid	Moles of residue per molecule of oxidized lysozyme*	Moles and percentages of residue detected as *N*-terminal**					
		Before digestion		15-min. digestion		24-hr. digestion	
		moles.	*%*	*moles.*	*%*	*moles*	*%*
Gly	11			0.15	1.4	0.53	4.8
Ala	10			0.54	5.4	0.79	7.9
Val	6			0.28	4.7	0.77	13
Leu+ ILeu	14			+		0.38	2.7
Ser	10	0.05	0.5	0.75	7.5	1.7	17
Thr	7			0.16	2.3	0.10	1.4
Phe	3			0.22	7.3	0.56	19
Tyr	3			±		±	
Glu Asp	17	0.10	0.6	0.80	4.7	2.3	14
Lys	6	0.10	1.7	0.14	2.3	0.13	2.2
Arg	11			+		+	
$CySO_3H$	10***			+		+	
Total moles of residues		0.25		3.0		7.3	

Signs of + and ± express qualitatively the presence and uncertain presence of DNP-amino acid, respectively.

* Amino acid composition of oxidized lysozyme was assumed to be the same as lysozyme of molecular weight 14,800 according to Fromageot (*29*) and Lewis *et al.* (*30*), except CySS, Met, and Try (*cf.* ***).

** Recovery factors for acid hydrolysis were adopted according to the literatures (*15, 31*).

Percentage of digestion in each residue which was adopted according to Desnuelle *et al.* (*9*) was calculated from moles of residue per mole of each amino acid residue of undigested molecule, and the actual extent of digestion of Ser, Glu + Asp, and Lys is the difference between each step and control.

*** Since it was suggested by Toennies *et al.* (*32*) that CySS was quantitatively converted to $CySO_3H$ by the performic oxidation, 5 moles of CySS in lysozyme might be converted to 10 moles of $CySO_3H$ in oxidized lysozyme.

of enzyme reaction. The extent of hydrolysis in each step, even in 24-hours' digestion, was cosiderably small, i.e., about 3 and 8 moles per oxidized lysozyme molecule in 15-minutes' and 24-hours' digestions, respectively. This might be attributed not only to the large ratio of substrate to enzyme but also to the low recovery of DNP-amino acid in the analytical process, such as acid hydrolysis and chromatography. DNP-Gly was so destroyed by acid hydrolysis that a far larger amount of this amino acid might actually exist as one of the *N*-terminal residues of peptides produced by digestion, than the amount detected. Water-soluble DNP-amino acids were not quantitatively identified, and di-DNP-His was scarcely found. For the reason mentioned above, completely quantitative results were not obtained, but it was concluded that Ser, Ala, Val, Glu+Asp and/or Glu(NH_2)+Asp(NH_2), Gly, and Phe are found as the *N*-terminal residues of the peptides produced from oxidized lysozyme by the action of BPN'.

2. *C-Terminal Residues in Digestion Product from Oxidized Lysozyme*—As shown in Table II, one mole of Leu+ILeu was found as the *C*-terminal residue per mole of undigested protein. If DNP-Leu+-ILeu fraction was occupied by only DNP-Leu, this result agrees with that obtained by Ohno (*27*) and Thompson (*28*). Ohno (*21*) also described that only one mole of Asp has a free β-carboxyl group and all other acidic amino acid residues are present in amide forms in lysozyme. Therefore, the former residue is extracted with sodium bicarbonate solution as α,α-di-DNP-Asp α-hydrazide. A control experiment on a paper chromatography of the sample without treatment by acid is shown in Fig. 2. There are spots of DNP-Leu, DNP, No. 1 (yellow), No. 2 (light brown), and others, and no DNP-acidic amino acids are found on the chromatogram. When spots No. 1 and No. 2 were extracted and hydolyzed with acid and the resulting hydrolyzate was again developed on paper, DNP-acidic amino acids appeared instead of the above two spots. When the control sample was hydrolyzed with acid, DNP-acidic amino acids, DNP-Leu, and DNP appeared on the paper chromatogram, but the other spots found in above were not detected. Hence, spots No. 1 and No. 2 are thought to be hydrazide-DNP derivatives of acidic amino acids. Though it was uncertain whether the spots were α-, β-, or γ-hydrazide derivatives, they were all regarded as α-hydrazide derivatives for the present*. Four moles of DNP-acidic amino acids

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* We have no information on the chromatograms of β- and γ-hydrazide derivatives on filter paper, but the spots No. 1 and No. 2 are probably α-hydrazide derivatives, because lysozyme has no Asp(NH_2) and Glu(NH_2) as the *C*-terminal residue (*27, 28*).

TABLE II

C-Terminal Residues in Digestion Porduct from Oxidized Lysozyme by the Action of BPN′

DNP-amino acid	Moles of residue per molecule of oxidized lysozyme*	Moles and percentages of residue detected as *C*-terminal**					
		Before digestion		15-min. digestion		24-hr. digestion	
		moles	%	*moles*	%	*moles*	%
Gly	11			0.28	2.5	0.47	4.3
Ala	10			0.61	6.1	1.1	11
Val	6			0.18	3.0	0.43	7.2
Leu+ ILeu	14	1.1	7.9	1.04	7.5	1.1	7.9
Ser	10			0.66	6.6	1.2	12
Thr	7			0.53	7.6	0.81	12
Phe	3			±		0.29	10
Tyr	3	+		+		+	
Glu+ Asp	17	4.0***	—	4.1	—	3.7	—
Lys	6			0.22	3.7	0.44	7.3
Glu+Asp (unhydrolyzed with acid)***	4			0.14	3.6	0.62	16
Total moles of residues		5.1		7.6		9.5	

* Amino acid composition is the same as shown in Table I.

** The values were obtained after acid hydrolysis.

Signs of + and ± have the same meanings as shown in Table I.

As recovery factors for hydrazinolysis and acid hydrolysis those in the literature (*20*, *15*, *32*) were adopted.

*** The values were calculated on assumption that some of the *C*-sides of 4 moles of Asp + Glu in the protein were hydrolyzed by the enzyme (see the text).

were detected after acid hydrolysis and it might be probably produced by the hydrolysis of amide bonds in the process of preparing the oxidized lysozyme.

Since ethyl acetate phase contains a large amount of hydrazide derivatives together with all di-DNP-Tyr and some part of di-DNP-His and DNP-Try, it was difficult to determine quantitatively even

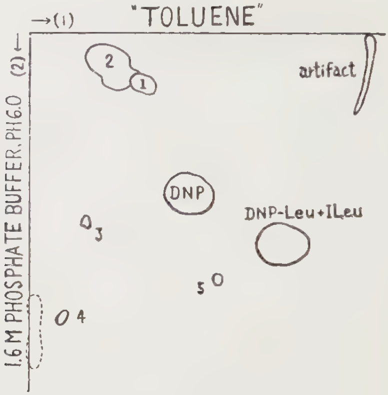

FIG. 2. Paper chromatogram of *C*-terminal residues of the control sample without treatment with acid.

Solvent systems were the same as in the method of Levy *et al.* (*22*, *23*).

Spots No. 1 and No. 2 moved into the place of DNP-Glu +-Asp (dotted line) and spots No. 3, No. 4, and No. 5 disappeared after acid hydrolysis of the sample.

after acid hydrolysis. Therefore, these amino acid residues were only shown whether they were present or not.

It was concluded from the result shown in Table II that mainly the *C*-sides of neutral and acidic amino acid residues in the protein were hydrolyzed by BPN′. Tyr was also found in each step, but the relative amount was approximately equal to one another and, even though the *C*-side of Tyr is attacked, the amount would probably be not so much.

The extent of hydrolysis of acidic amino acid residues, namely, 3,6 per cent and 16 per cent for 15-minutes' and 24-hours' digestion, respectively, was calculated on the assumption that 4 acidic amino acid residues were free in β- and γ-carboxyl groups. They were concluded from the supposition that the spots No. 1 and No. 2 in Fig. 2 were α-hydrazide derivatives of acidic amino acids. As mentioned above, when the amide or peptide bonds of Asp(NH_2) and Glu(NH_2) were hydrolyzed by the proteinase, the amount of DNP-Asp and -Glu should increase in the extract by treatment with acid in comparison with the control. However, in the present experiment, the amount of DNP-Asp and -Glu in each step was approximately equal to that of the control after acid hydrolysis and it was concluded that the amide or peptide bonds of Asp(NH_2) and Glu(NH_2) were not hydrolyzed by the enzyme.

3. *N- and C-Terminal Residues in the Digestion Product from Denatured Bacterial α-Amylase by BPN′*—Denatured bacterial α-amylase was digested for 24 hours by BPN′ under the same conditions as in the case of oxidized lysozyme, and *N*- and *C*-terminal residues of the digestion product were determined similarly as above. As the *N*-terminal residues were found Gly 5–6, Ala 4–5, Val 1–2, Ser 1–2, Thr 1–2, Glu+Asp and/or Glu(NH_2)+Asp(NH_2) 4–5 moles, and small amounts of Phe, Leu+ILeu, Tyr, and Lys per protein molecule, and as *C*-terminal resideues were found Gly 1–2, Ala 3, Val 2–3, Ser 1, Glu+Asp 2–3 moles, and small amounts of Thr, Phe, and Tyr per molecule.

4. *N-Terminal Residues in the Autodigestion Product of BPN′* (*2*)—About 40 mg. of BPN′, recrystallized 4 times, was dissolved in a small amount of sodium bicarbonate buffer solution of pH 8.5. The solution was mixed with FDNB dissolved in ethanol, vigorously shaken for about 2 hours at room temperature, and the *N*-terminal residues of the resulting product were analysed as above. Ser 1, Ala 1–2, Gly 1, Val 1, and Glu+Asp and/or Glu(NH_2)+Asp(NH_2) 1 moles were detected per protein molecule, taking the molecular weight as 30,000.

It might be concluded from the above results that the crystalline bacterial proteinase BPN′ has a broad specificity and hydrolyses *N*- and *C*-sides of neutral and acidic amino acid residues generally and of aromatic and basic amino acid residues partially in the proteins.

SUMMARY

The specificity of crystalline bacterial proteinase has been investigated by the fluorodinitrobenzene and hydrazinolysis-DNP methods. Oxidized lysozyme was mainly used as the substrate and other proteins, such as bacterial α-amylase and proteinase itself, were also used. It was found that this proteinase has a broad specificity and mainly attacks both *N*- and *C*-sides of neutral and acidic amino acid residues and others partially in the proteins.

The authors express their many thanks to Prof. S. Akabori, Dr. K. Ohno, Mr. T. Ikenaka, and Mr. I. Haruna for the kind technical guidance and to Mr. T. Seki for his kind advice on the technique of ion exchange resin chromatography and also to Nagase Co. Ltd., for the kind supply of the crystalline bacterial proteinase.

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The Journal of Biochemistry, Vol. 43, No. 6, 1956

ON THE SO-CALLED SIALIC ACIDS OF BLOOD CELLS AND SERUM

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(Received for publication, August 18, 1956)

In the previous paper, isolation of a crystalline reducing substance named ' sero-lactaminic acid ' from equine serum (*1*) was reported, which was closely related to sialic acid (2, 3), acetyl-neuraminic acid (*4*), lactaminic acid (*5*) or gynaminic acid (*6*).

At about the same time, Dr. Odin (*7*) isolated probably the same substance from human serum and claimed it as identical with sialic acid previously obtained from sheep submaxillary gland mucin (*8*) by means of x-ray diffraction pattern.

In 1951, Yamakawa and Suzuki (*9*) reported the presence and isolation of hemataminic acid (later confirmed as methoxyl neuraminic acid) from hematoside, a glycolipid of equine blood cells, and discussed that it would be present in the lipid probably as *N*-acetylated form. Two years later, Klenk and Lauenstein (*10*) reported in their reinvestigation of our work, that hydrolysis of hematoside produced no volatile acid, and denied the presence of acetyl group. More recently, Blix and his co-workers (*8*) reported bovine submaxillary sialic acid contains an *N*-acetyl and an *O*-acetyl group, the latter being very easily split off, whereas the ovine (sheep) substance has only an *N*-acetyl, and the porcine (hog) acid no acetyl but an *N*-glycolyl group, instead.

Moreover, Svennerholm (*11*) isolated sialic acid from brain ganglioside, later, Blix and Odin (*12*) stated it would be the same as sialic acid of sheep submaxillary mucin, which would probably be identical with *N*-acetyl neuraminic acid.

In the course of the studies of influenza virus hemagglutination receptor, Klenk and his collaborators (*13*, *14*), Gottschalk (*15*) and Blix's school (*16*) became in agreement in regarding the receptor site to be correlated with so-called sialic acid. It was found in this laboratory the mucopolysaccharide fraction (90 per cent phenol extract) of delipidized human blood stroma, although not yet satisfactorily purified, has a marked capacity to inhibit viral hemagglutination and contains a considerable amount of Bial-chromogen besides hexosamine (*17*, *18*).

The structure of hemataminic acid and of sialic acid were suggested by the present author (*19*) and recently by Gottschalk (*20*, *21*), but available evidences were not sufficient to exclude other structures.

Thereafter, the present author has regarded the formula previously assumed for hemataminic acid as inadequate, because it could not explain his recent finding that formalin (as dimedon) was produced after periodate oxidation (unpublished).

In a mean while, Klenk and his co-workers (*22*) have recently pointed out this erroneous assumption and presented a probable structure for neuraminic acid.

In this report, delipidized human as well as equine stromata, hematoside and human serum were treated with dilute sulfuric acid to release Bial-chromogen (=sialic acids) and the chromogen was purified by adsorption on ion exchanger just in the same way as described in the case of sero-lactaminic acid from equine serum (*1*).

In each case, it was succeeded to obtain crystalline needles. The materials from hematoside as well as delipidized equine stroma gave no volatile acid on hydrolysis and available evidences show the amino group was substituted with glycolyl group as in case of porcine submaxillary sialic acid (*11*) ; on the contrary, the substance from human delipidized stroma and human serum contained the same amount of acetyl group as equine serum sialic acid (=serolactaminic acid) (*1*). While the dried sample from human stroma gave no methoxyl value despite after crystallization from methanol-ether, the acid from serum contained a methoxyl group even after drying over phosphorus pentoxide at elevated temperature in a high vacuum. On the other hand, the serum-sialic acid crystallized from water-acetoneether instead of usual methanol-ether solvent system was devoid of methoxyl and remained methoxyl-free even after retreatment with methanol ; although the serum-sialic acid once crystallized from methanol-ether was not converted to methoxyl-free compound after recrystallization from water-acetone and drying under high vacuum at elevated temperature. This methoxyl value of serum sialic acid is now proved to be due to labile methyl ester formation during prolonged treatment with methanol and not to crystal solvent (*1*), because its aqueous solution is neutral and cannot be titrated electrometrically at low temperature.

From the results of repeated elemental analyses of these sialic acids, particularly from their nitrogen values, the author is fully assured that $C_9H_{17}NO_8$ is more adequate than $C_{10}H_{19}NO_9$ for prehemataminic acid or neuraminic acid. While Klenk and some other workers prefer 10-C for neuraminic acid to 9-C formula, the present author consistently adopts the latter. Blix's school (*11*) and Gottschalk (*20*, *21*) fall in with this opinion. It is supposed that lower nitrogen value reported by Klenk might be due to the application of Kjeldahl's wet combustion method, whereas Pregl's dry combuation has been used in this laboratory.

It is a matter of peculiar interest to note a correlation between viral hemagglutination and the nature of sialic acids; namely, the well-known α-inhibitors such as serum mucoprotein, sheep submaxillary mucin or human erythrocytes extract all contain *N*-acetylated form of sialic acid, whereas the equine erythrocytes extract which is devoid of inhibitory activity has no *N*-acetyl- but *N*-glycolyl compound.

In this connection, the report of Curtain and Pye (*23*) that bovine submaxillary mucoprotein shows the restricted inhibitory action against influenza virus hemagglutination might be suggestive.

EXPERIMENTAL

General Isolation Procedure of Sialic Acids

Two hundred grams of delipidized blood stroma or one l. of serum were heated with dilute sulfuric acid at pH 1–2 for one hour. After cooling, it was dialysed against successive changes of 3 l. of deionized water. The dialysate was made free from sulfuric acid with baryt, concentrated in vacuo to about 100 ml. and passed through Amberlite IRA 400 (OH phase), on which nearly total amount of Bial chromogen adsorbed. After washing the column with water, the chromogen was eluted with 200 ml. of *N*-sulfuric acid. The eluate was neutralised with baryt, the filtrate was concentrated to a small volume, and chloride present was removed with silver sulfate, then sulfate successively with baryt. The resulting fluid was passed through Dowex 50 (H phase) column and the effluent was concentrated and lyophilized. The nearly colorless fluffy material was in some cases dissolved in methanol, to which were added gradually ether and petroleum ether to faint turbidity. On standing in an ice-box, crystalline needles separated out usually in a few days. In other cases, the fluff was dissolved in a small volume of water, to which acetone and ether were gradually added. Colorless crystalline needles appeared usually after a few days.

Analyses

All the samples were dried in a high vacuum at 90° over P_2O_5 until constant weight. C, H and N were determined by Pregl's dry combustion method.

Human Blood Stroma Sialic Acid

Yield: 175 mg. from 100 g. stroma.
Decomp. p. 188°
Found C 42.97, 43.36, 43.21; H 6.24, 6.25, 6.23; N 4.71; OCH_3 0; CH_3CO 14.11
Calcd. for *N*-acetyl-prehemataminic acid,
$C_{11}H_{19}NO_9$: C 42.72, H 6.19, N 4.53, CH_3CO 13.91
$C_{12}H_{21}NO_{10}$: C 42.48, H 6.23, N 4.13, CH_2CO 12.70

Equine Blood Stroma Sialic Acid

Yield: 123 mg. from 100 g. stroma.
Decomp. p. 188–9°.
Found C 41.20, 40.48, 41.16; H 5.83, 5.99, 5.96
N 4.45, 4.42, 4.55; OCH_3 0, CH_3CO 0.
NH_2-N (by van Slyke's manometric method) 0.
Calcd. for *N*-glycolyl-prehemataminic acid,
$C_{11}H_{19}NO_{10}$: C 40.61: H 5.84; N 4.31
$C_{12}H_{21}NO_{11}$: C 40.56; H 5.91; N 3.94
$[\alpha]_D^{20} = -29.8°$ (c=5.6, water)

Sialic Acid from Hematoside

1.2 g. of hematoside yielded 46 mg. of crystalline sialic acid, decomposing at 181°.

Found C 40.59; H 6.15; N 4.50

Human Serum Sialic Acid

Lyophilized and delipidized human serum was used as the starting material. Two hundred g. of dry serum residue yielded about 140 mg. of crystalline sialic acid.

(A) Sample crystallized from water-acetone-ether.
Decomp. p. 187–8°.
Found C 42.95; H 6.08; N 4.66; OCH_3 0
$[\alpha]_D^{20} = -30°$ (c=3.3, water)

(B) Sample crystallized from methanol-ether-petroleum ether.
Found C 43.81, 43.73, 44.01; H 6.50, 6.42, 6.50; N 3.93, 3.86; OCH_3 8.73; CH_3CO 12.18
Calcd. for $C_{13}H_{23}NO_{10}$, C 44.19; H 6.51; N 3.96; OCH_3 8.78; CH_3CO 12.18

Equine Serum Sialic Acid (Sero-Lactaminic Acid (1))

(A) Sample crystallized from water-acetone-ether
Found C 42.49; H 6.24; N 4.45; OCH_3 0

This methoxyl-free sample was recrystallized from methanol-ether-petroleum ether.
Found C 42.61, 41.89; H 6.07, 6.15; N 4.59

(B) Sample crystallized from methanol-ether (already reported in the previous paper (*1*) as sero-lactaminic acid.)

Found C 42.2–43.5; H 6.5–6.9; N 3.9–4.4; OCH_3 8.5–9.0
Calcd. for $C_{12}H_{23}NO_{10}$–CH_3OH: C 42.2; H 6.74; N 4.10;
OCH_3 12.54; CH_3CO 13.2

This methoxyl-containing sample was recrystallized from water-acetone-ether.

Found C 43.54; H 6.58; OCH_3 8.19

Paper Chromatography of Amino-substituted Organic Acids Combined in Sialic Acids

Paper chromatography was carried out according to the technique of Reid and Lederer (*24*). Sialic acids samples were heated on a steam-bath with 2 *N*-sulfuric acid. After cooling, the hydrolysates were made alkaline with baryt, filtered and passed through Dowex 50 column to remove cations. A few drops of ammonia were added and the solutions were evaporated *in vacuo* to dryness. The residue was dissolved in a small amount of water and chromatographed on Toyo filter paper No. 50 using developing solvent system of NH_3-saturated *n*-butanol. After 18 hours development the position of acids were indicated by spraying bromocresol purple containing formalin and holding in an atomosphere of ammonia. As shown in Fig. 1, in case of equine stroma sialic acid an intense spot was indicated at the position of glycolic acid but less intense spot corresponding to acetic acid was also seen. In case of serum sialic acid as well as human stroma sialic acid, strong acetic acid spot and weak glycolic acid spot were indicated. Moreover, especially in case of human serum sialic acid, a weak but distinct spot appeared at the position faster than acetic acid.

Another trial to detect glycolic acid was carried out according to the procedure of Star,, Goodban and Owens (*25*) using their Solvent D: Benzyl alcohol 50, *n*-butanol 50, water 10 and formic acid (85 per cent) 1. A spot corresponding to glycolic acid was indicated.

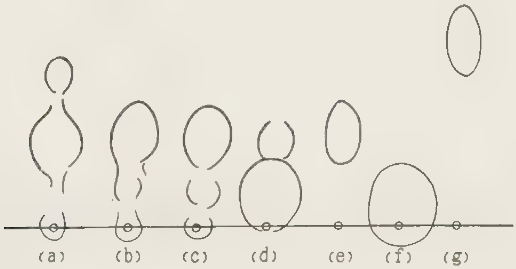

FIG. 1. Paper chromatogram of organic acids in sialic acids hydrolysates according to the method of Reid and Lederer.

(a) Human serum, (b) equine serum, (c) human stroma, (d) equine stroma, (e) acetic, (f) glycolic, (g) propionic acid.

Column Chromatography of Organic Acids

The procedure used in this study generally followed the method of Klenk and Bongard (*26*) using silicic acid column technique on Säule D.

In a mortar, 5 g. of silicic acid (Mallinckrodt, A. R.; suitable for chromatographic analysis) and 3 ml. of 0.5 *N* sulfuric acid were thoroughly mixed, to which 40 ml. of 1 per cent methanol-containing chloroform was gradually added while stirring. The resulting silica gel was introduced to a tube (0.9 × 50 cm.) and chromatography was performed with the acid hydrolysates of sialic acid samples. Caution was paid at this stage to avoid vacuum concentration in acidic reaction. Elution was carried out at first with 120 ml. of 1 per cent methanol-containing chloroform and then 200 ml. of 20 per cent methanol containing chloroform. Exactly 4 ml. of effluent was taken with an automatic fraction collector. The measurement of acids was carried out with *N*/100 methanolic NaOH using phenolphthalein as an indicator.

Contrary to paper chromatographic results, the pattern obtained showed only one peak in each case. In case of equine stromic sialic acid, a peak corresponding to glycolic acid, and in case of human serum sialic acid a peak of acetic acid were indicated. (Fig. 2).

Fig. 2(A)

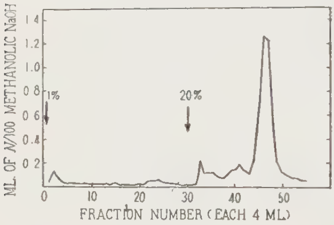

Fig. 2(B)

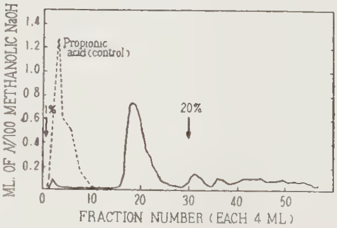

Fig. 2, A. Column chromatography of organic acids in equine stromic sialic acid hydrolysate. Main peak: glycolic acid.

Fig. 2, B. Column chromatography of organic acids in human serum sialic acid hydrolysate. Main peak: acetic acid.

SUMMARY

1. From human and equine delipidized blood stroma, acetylated form and glycolated form of sialic acids, were obtained, respectively.
2. Acetylated form of sialic acid was also obtained from human and equine serum.
3. The analysis of these substances was presented.

The author wishes to thank Mr. B. Kurihara and Miss R. Ota for C, H and N analyses.

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ADDENDUM

Oxidation of Sialic Acid with Hypoiodite—To 500 mg. of equine stromic sialic acid were added 0.1 N I–KI (100 ml.) and 0.1 N NaOH (200 ml.). After 30 minutes, the solution (Bial-negative) was completely deionized and concentrated, yielding 230 mg. of syrupy material. After hydrolysis with 2 N sulfuric acid and subsequent deionisation, a crystalline amino acid was obtained. Yield: about 100 mg. It charred at 215°.

Analysis: C 39.89∼40.52; H 5.68∼6.23; N 4.95∼5.18
Calcd. for $C_9H_{17}NO_9$: C 40.75, H 5.66, N 5.28

Klenk *et al.* (*22*) attempted but failed to obtain a crystalline material by the same procedures but reported the presence of 0.86 mol. of α-amino nitrogen as the results of heating of its aqueous solution with ninhydrin. However, the crystalline amino acid gave no CO_2 at pH 2.5 by heating with ninhydrin. Qualitative ninhydrin reaction was only positive after adding a drop of alkali just as in case of hemataminic acid (*9*). The fact that crystalline 9-carboned amino acid was obtained would hardly be explained by α-keto or α-hydroxy acid structure of Gottschalk (*20*). In view of analysis, this oxidation product seems now most probable to be an acid amino lactone.

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